UNIVERSITA' DEGLI STUDI DI ROMA "SAPIENZA"

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Reversible Immortalization and Transfer of a Dystrophin Human Artificial Chromosome into Human Mesoangioblasts: Towards Autologous Cell Therapy of Duchenne Muscular Dystrophy

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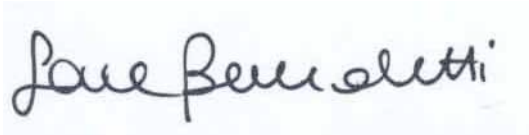

DECLARATION

This thesis has been written by myself and has not been used in any previous application for a degree. All the results presented here were obtained by me in collaboration with researchers of my laboratory.

A manuscript containing part of this results is in preparation and will be submitted for publication in the very next months.

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1. ABSTRACT

Human mesoangioblasts (MABs) are vessel–associated progenitors able to differentiate into skeletal muscle and to reconstitute the damaged muscle fibers when injected into a Duchenne Muscular Dystrophy (DMD) mouse model (*mdx*), an incurable myopathy for which no therapy currently exists. Because of these features, human MABs are now entered clinical experimentation based upon allogeneic transplantation. However autologous cell therapy for DMD still faces significant limitations, first of all the large size of the dystrophin gene that hampers its allocation into conventional gene-delivery tools such as viral vectors.

In order to overcome these limitations, a novel approach of autologous cell therapy to treat DMD was developed by transplanting dystrophic MABs genetically-corrected with a Human Artificial Chromosome (HAC) containing the entire dystrophin locus (DYS-HAC). The feasibility of this strategy was already proved: MABs derived from *mdx* mouse and genetically corrected with the DYS-HAC, were able to engraft skeletal muscle and restore dystrophin positive fibers in dystrophic mice, resulting into a significant morphological and functional amelioration of the phenotype.

In the case of DMD MABs, an additional step of immortalization is fundamental before DYS-HAC transferring, which requires clonal expansion and analysis, since they undergo replicative senescence. To reversibly immortalize normal and dystrophic human MABs, lentiviral vectors encoding floxed hTERT IRES-HSV1-TK and Bmi-1 have been used.

Normal clones have been characterized for proliferation and proper expression of hTERT and Bmi-1; they remained growth factor-dependent, contact-inhibited,

non tumorigenic and myogenic *in vitro*. Notably, upon transplantation into dystrophic mice, they engrafted skeletal muscle and restored dystrophin expression. I then moved to the immortalization of DMD MABs in order to transfer the DYS-HAC, obtaining immortalized DMD DYS-HAC MABs. These data set the conditions for future clinical translation of this experimental strategy for the treatment of the DMD patients.

2. INTRODUCTION

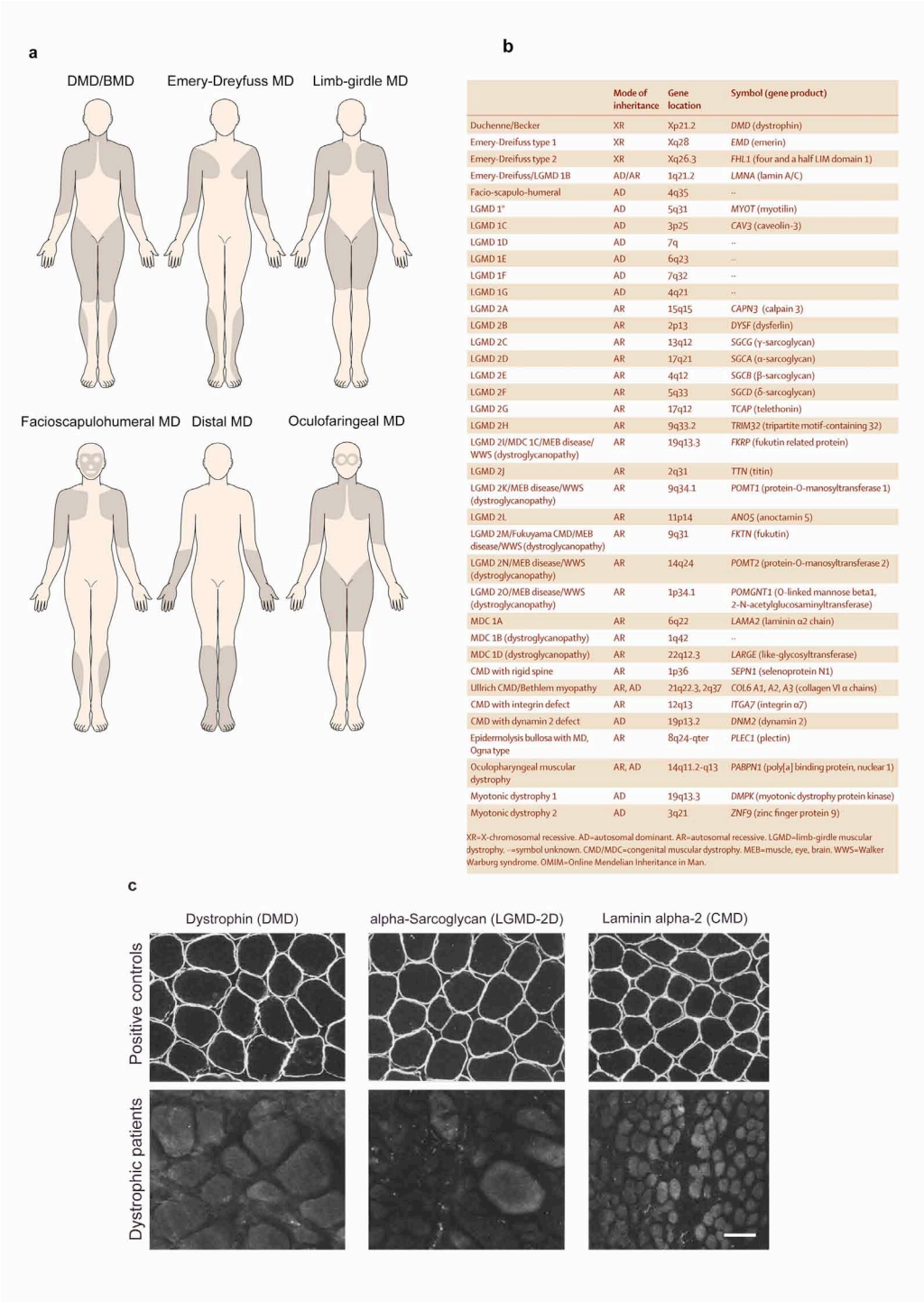
2.1 THE MUSCULAR DYSTROPHIES

2.1.1 Clinical presentation

Muscular dystrophies (MDs) are inherited neuromuscular disorders characterized by progressive muscle wasting and weakness leading to a wheelchair constriction and to a heart and/or respiratory failure in the most severe forms (Emery, 2002). MDs are all well defined pathologies recognized by drastic changes into common histological features such as variation in muscle fiber size, muscular fibers degeneration and regeneration with replacement of muscle by scar and fat. Nevertheless, they displayed a great heterogeneity due to the genetic basis, the clinical onset and the progress and the severity of the pathology (Manzur and Muntoni, 2009). For all these reasons, MDs can be divided into several groups, mainly in accordance with the anatomical distribution of muscle weakness: Duchenne and Becker (predominant proximal major involvement); Emery-Dreifuss; distal; oculopharyngeal; facioscapulo-humeral (FSHD); limb-girdle (LMGD, which is the most heterogeneous group) (Figure 2.1.1.1). In several forms there is a cardiac involvement; additionally, some MDs, such as DMD, could also involve the central nervous system (where dystrophin is also expressed) resulting in a variable cognitive impairment (Emery, 2002).

Both DMD and Becker MD (BMD) are X-linked disorders caused by mutations in the gene encoding the sarcolemmal protein dystrophin, an integral part of a complex that links the intracellular cytoskeleton with the extracellular matrix (Muntoni et al., 2003). DMD symptoms start in early childhood, with

difficulties mainly in running and climbing stairs; frequent enlarged calves, toe/waddling gait and Gower's manoeuvre (a child climbs up his thighs, pushing down on them, to extend the hips and trunk), in addition to a frequent global developmental delay (Essex and Roper, 2001) are signs which can permit a easy recognition. An almost unequivocal marker for DMD diagnosis is the elevated level (10–100X normal, since birth) of serum creatine kinase (CK) (Manzur and Muntoni 2009). Weakness is initially proximal and progressively leads patients to be wheelchair bound by age 11-15. Pneumonia, and cardiac failure are the most frequent cause of death that usually occurs in the late 20s. In BMD the distribution of muscle wasting and weakness resembles DMD, but generally the disease is less severe, with some patients living without any symptoms until late in life. Dystrophin is normally absent in patients with DMD, whereas is reduced in amount or abnormal in size in patients with BMD (Monaco and Kunkel, 1988): this reflects the type of mutation in the dystrophin gene, as deletions that do not prevent synthesis of dystrophin amino and carboxy terminal domains (i.e. in frame deletions) lead to BMD (Muntoni et al., 2003). However, in rare DMD cases, dystrophin can be detected, or is occasionally undetectable in mild cases of this disorder (Hattori et al., 1999). Clinical diagnosis is validated by immunohistochemistry, immunoblotting and molecular analysis of the specific mutation (the most common being intragenic deletions, which account for 65% of dystrophin mutations).



	Mode of inheritance	Gene location	Symbol (gene product)
Duchenne/Becker	XR	Xp21.2	*DMD* (dystrophin)
Emery-Dreifuss type 1	XR	Xq28	*EMD* (emerin)
Emery-Dreifuss type 2	XR	Xq26.3	*FHL1* (four and a half LIM domain 1)
Emery-Dreifuss/LGMD 1B	AD/AR	1q21.2	*LMNA* (lamin A/C)
Facio-scapulo-humeral	AD	4q35	..
LGMD 1°	AD	5q31	*MYOT* (myotilin)
LGMD 1C	AD	3p25	*CAV3* (caveolin-3)
LGMD 1D	AD	7q	..
LGMD 1E	AD	6q23	..
LGMD 1F	AD	7q32	..
LGMD 1G	AD	4q21	..
LGMD 2A	AR	15q15	*CAPN3* (calpain 3)
LGMD 2B	AR	2p13	*DYSF* (dysferlin)
LGMD 2C	AR	13q12	*SGCG* (γ-sarcoglycan)
LGMD 2D	AR	17q21	*SGCA* (α-sarcoglycan)
LGMD 2E	AR	4q12	*SGCB* (β-sarcoglycan)
LGMD 2F	AR	5q33	*SGCD* (δ-sarcoglycan)
LGMD 2G	AR	17q12	*TCAP* (telethonin)
LGMD 2H	AR	9q33.2	*TRIM32* (tripartite motif-containing 32)
LGMD 2I/MDC 1C/MEB disease/ WWS (dystroglycanopathy)	AR	19q13.3	*FKRP* (fukutin related protein)
LGMD 2J	AR	2q31	*TTN* (titin)
LGMD 2K/MEB disease/WWS (dystroglycanopathy)	AR	9q34.1	*POMT1* (protein-O-manosyltransferase 1)
LGMD 2L	AR	11p14	*ANO5* (anoctamin 5)
LGMD 2M/Fukuyama CMD/MEB disease/WWS (dystroglycanopathy)	AR	9q31	*FKTN* (fukutin)
LGMD 2N/MEB disease/WWS (dystroglycanopathy)	AR	14q24	*POMT2* (protein-O-manosyltransferase 2)
LGMD 2O/MEB disease/WWS (dystroglycanopathy)	AR	1p34.1	*POMGNT1* (O-linked mannose beta1, 2-N-acetylglucosaminyltransferase)
MDC 1A	AR	6q22	*LAMA2* (laminin α2 chain)
MDC 1B (dystroglycanopathy)	AR	1q42	..
MDC 1D (dystroglycanopathy)	AR	22q12.3	*LARGE* (like-glycosyltransferase)
CMD with rigid spine	AR	1p36	*SEPN1* (selenoprotein N1)
Ullrich CMD/Bethlem myopathy	AR, AD	21q22.3, 2q37	*COL6 A1, A2, A3* (collagen VI α chains)
CMD with integrin defect	AR	12q13	*ITGA7* (integrin α7)
CMD with dynamin 2 defect	AD	19p13.2	*DNM2* (dynamin 2)
Epidermolysis bullosa with MD, Ogna type	AR	8q24-qter	*PLEC1* (plectin)
Oculopharyngeal muscular dystrophy	AR, AD	14q11.2-q13	*PABPN1* (poly[a] binding protein, nuclear 1)
Myotonic dystrophy 1	AD	19q13.3	*DMPK* (myotonic dystrophy protein kinase)
Myotonic dystrophy 2	AD	3q21	*ZNF9* (zinc finger protein 9)

XR=X-chromosomal recessive. AD=autosomal dominant. AR=autosomal recessive. LGMD=limb-girdle muscular dystrophy. ·=symbol unknown. CMD/MDC=congenital muscular dystrophy. MEB=muscle, eye, brain. WWS=Walker Warburg syndrome. OMIM=Online Mendelian Inheritance in Man.

Figure 2.1.1.1 - The muscular dystrophies.

(a) Muscular groups predominantly affected in different forms of MDs. **(b)** Inheritance of the MDs. **(c)** Immunofluorescence staining of skeletal muscle sections of three different MD patients showing absence of sarcolemmal signal of dystrophin, alpha-sarcoglycan and laminin-alpha2. Adapted from Emery, 2002 and Bushby, 2009 (Bushby et al., 2009).

2.1.2 Duchenne Muscular Dystrophy: Genetic and Pathophysiology

The genes and their protein products that are responsible of the majority of muscular dystrophies have now been identified. This happened more than twenty years ago both for DMD and BMD, when they were described as X-linked recessive genetic disease caused by mutations in the dystrophin gene located at locus Xp21 (Kunkel et al., 1985; Ray et al., 1985). Dystrophin is a rod-shaped protein which is supposed to be fundamental to protect sarcolemma from continuous and subsequent contractions, being the source of an indirect link between the cytoskeletal actin and the intermediate filaments in the muscle fiber with the extracellular matrix; as a matter of fact, the amino-terminal end binds to the cytoskeletal actin whereas the carboxyl-terminal end binds to the dystrophin associated glycoprotein complex (DGC) at the sarcolemma (Figure 2.1.2.1).

Generally, the mutations in the dystrophin gene caused alteration of the reading frame, resulting in a severe reduction or absence of dystrophin in the skeletal and cardiac muscles, which turn into DMD (or BMD). In addition, 65% of DMD patients have intra-genic out-of-frame deletions and approximately 10% have duplications of one or more exons of the dystrophin gene. The remaining patients have point mutations or smaller gene rearrangements such as insertions of repetitive sequences, splice site mutations and intronic deletions. In general, out-of-frame mutations give rise to a strong reduction or a complete absence of dystrophin resulting into DMD phenotype, whereas in-frame mutations lead to the expression of a partially functional truncated protein, resulting in the milder form, the BMD. Nevertheless, in-frame mutations in actin and dystroglycan binding domains, which plays a crucial role for dystrophin function, can lead to a DMD phenotype, while some out-of-frame mutations are associated with BMD. It

is also important to remember that there is a high incidence of *de novo* mutations (Manzur and Muntoni 2009), which will make eradication of the disease by genetic counselling impossible. Absence/reduction in dystrophin expression causes disruption of the DGC and, as a consequence leads to an increased sarcolemma fragility that results in contraction-induced breakdown of muscle membrane, high levels of intra-cytoplasmatic calcium, followed by muscle fiber damage and tissue degeneration. Several dystrophin isoforms are also expressed in brain and heart, and their deficiency in these tissues is responsible respectively for mental impairment (though this had been questioned) and cardiomyopathy, which hang up with the muscular phenotype in approximately a third of cases.

Currently there is no yet any efficacious treatment for muscular dystrophies. Since DMD is surely the most severe form, the majority of the efforts are focused on the management of this disease (Bushby et al., 2010a, 2010b). Glucocorticoids remain the standard drug administered to dystrophic patients: it partially ameliorates symptoms and delays the progression of the disease (Muntoni et al., 2002). Respiratory care and treatment of cardiac complications is critical in later stages of disease, together with surgical correction of contractures, which might be helpful when walking and posture become difficult.

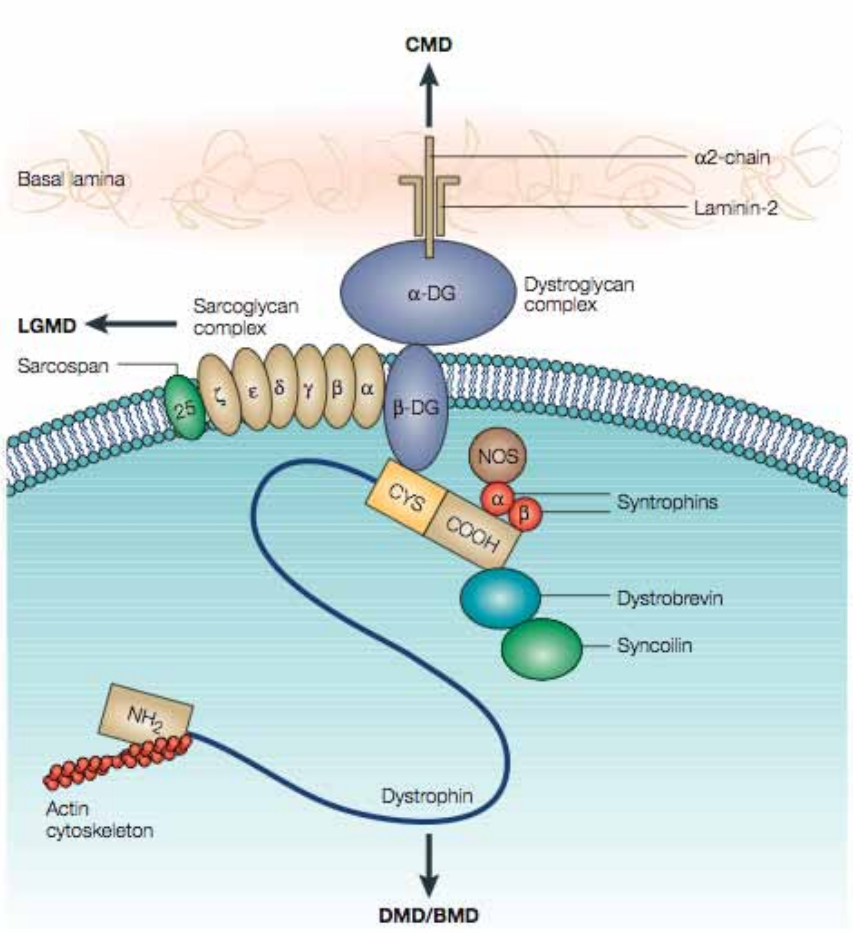


Figure 2.1.2.1 - Dystrophin-glycoprotein complex (DGC) organization. Schematic representation of the organization of DGC and aetiology of muscular dystrophies. Dystrophin interacts with cytoplasmic trans-membrane and extracellular protein in skeletal muscle. Mutation in the dystrophin and other members of DGC give rise to a variety of muscular dystrophies. BMD: Becker Muscular Dystrophy; DMD: Duchenne Muscular Dystrophy; CMD: Congenital Muscular Dystrophy; CYS: Cysteine; DG: Dystroglycan; LGMD: Limb Girdle Muscular Dystrophy; NOS: Nitric Oxide Sinthase. Taken from Khurana and Davies, 2003 (Khurana and Davies, 2003).

2.1.3 Experimental therapies

2.1.3.1 Pharmacological therapy

The main aim of drug experimental therapies is not to definitively cure but to ameliorate the dystrophic phenotype by decreasing inflammatory process, improving calcium homeostasis, increasing survival, commitment and proliferation of myogenic progenitors. Taken as a whole, the pharmacological approaches could provide an immediate and suitable therapeutic opportunity to ameliorate the life quality of DMD and other MDs patients and, most importantly, to delay the pathology progression with the perspective that, in time, other therapies may become available.

Since the degeneration of skeletal muscle fibers is accompanied by chronic inflammation, which is finally responsible of sclerosis and of a reduced vascularisation (Deconinck et al., 1997), the main pharmacological approach concerns the use of corticosteroids (Muntoni et al., 2002; Manzur et al., 2004) that were responsible of a short term modest but significant reduction of inflammatory and immunological responses. A big deal of other drugs (Mozzetta et al., 2009) are now under pre-clinical or clinical experimentation: inhibitors of negative myogenic regulators such as myostatin and TGF-β signaling pathway (Bogdanovich et al., 2002); other anti-inflammatory molecules (Muntoni et al., 2002) among which the Nitric oxide (NO) (Brunelli et al., 2007); IGF1 (insulin growth factor 1), able to activate satellite cells and to increase muscle size (Musaro et al., 1999; Barton et al., 2002); chromatin modifying agents such as HDACs, responsible of an increase expression of genes involved into regeneration process such as follistatin (Minetti et al., 2006). Among these strategies, myostatin-neutralizing antibodies underwent clinical experimentation

but failed (Wagner et al., 2008). This is an indication that novel approaches are still needed.

Other compounds, such as angiotensin-receptor blocker, can ameliorate the dystrophic phenotype of *mdx* mice (Wagner et al., 2008). Up-regulation of utrophin to counteract the loss of dystrophin in DMD is another interesting approach, and at that moment different studies are testing recently identified transcriptional activators of utrophin (Miura and Jasmin, 2006). Another approach targets mitochondria, inhibiting cyclophillin D, has proven to lead to benefits in dystrophic mouse models (Miura and Jasmin, 2006).

2.1.3.2 Gene therapy

The final aim of gene therapy is to replace or fix the mutated gene with a correct and functional form, thus reverting the pathological phenotype. The situation is particularly complicated for skeletal muscle genetic diseases: first, skeletal muscle is the most abundance tissue of the body and, therefore, there is the need to replace the mutated gene into a huge number of post-mitotic nuclei; second, in the case of DMD, the involved gene, dystrophin, is the largest gene of the human genome (entire genetic locus 2.4 Mb, cDNA 14kb), so it is difficult, or better impossible, to accommodate it into any classical delivery vector. For this reasons, a deal of different gene delivery systems has been tested and most of them are now under laboratory or clinical experimentation.

Viral vectors

DNA-based vectors have great potential for MD gene therapy. In particular adenoviral vectors have the peculiar ability of carrying up to 30kb of transgenic

DNA and to this purpose they have been largely used to deliver target genes to muscle (Welch et al., 2007). On the other hand their non-integrating nature, together with high immunogenicity, especially in the case of first generation vectors, the tendency to be silenced and the difficulty, in some cases, to cross skeletal muscle fibers strongly limit their use as gene delivery tools (Chen et al., 1997; DelloRusso et al., 2002).

Over the past decade, the generation of a new class of recombinant adeno-associated virus (rAAV) exhibiting a unique skeletal muscle tropism has provided significant progress in this direction (Blankinship et al., 2004; Asokan et al., 2010). Of particular interest is the combination of rAAV delivery strategy with truncated dystrophin small enough to be accommodated within a rAAV (Harper et al., 2002; Watchko et al., 2002). Systemic delivery of a truncated microdystrophin through AAV has been successful in mice (Gregorevic et al., 2004), but the delivery of vector to all striated muscle in a larger organism still presents a challenge and evidence of rAAV efficacy in a large dystrophic animal model is now missing. Additionally, as reported for adenoviral vector, the immunogenicity of the rAAV capsid is a significant obstacle to long-term and repeated transductions of skeletal muscle (Yuasa et al., 2007). To overcome this problem, a transient immunosuppression (Wang et al., 2007) as well as intravenous rAVV delivery into neonatal dogs have been tested (Yue et al., 2008); even if results are encouraging, additional evidences will be necessary to confirm these results. At the moment, AAV-mediated delivery of α-sarcoglycan to dystrophic mice has been shown to be successful (Pacak et al., 2007) and clinical studies in LGMD patients are in progress (Rodino-Klapac et al., 2008).

Another common way to deliver the therapeutic gene is the use of retrovirus-based vectors, such as HIV-derived lentiviral vectors but importantly, they cannot deliver more than 8kb of exogenous DNA. At difference with previously employed retroviral vectors, lentiviral ones are able to transduce non-mitotic cells so, together with their lower tumorigenic potential related to insertional mutagenesis, they are interesting tool for therapeutic genes. Recently, integration-deficient lentiviral vectors have been generated which have a much lower risk of insertional mutagenesis than integrating lentiviral vectors (Wanisch and Yanez-Munoz, 2009; Banasik and McCray, 2010). Although integration-deficient lentiviral vectors can mediate stable expression in non-dividing cells, they show transient expression in proliferating cells. This last issue still raises some concerns for their clinical use and the limited space for transgenes makes them unsuitable for large cDNAs, such as dystrophin. Integrating lentiviral vectors have been so far predominantly used for the *ex vivo* transduction of various types of stem cells, in order to use them for cell therapy studies in dystrophic animal models.

Non-viral vectors: human artificial chromosomes (HACs)

Although a large number of different approaches have been attempted to achieve efficient gene transfer and long-term gene expression, this challenging task remains unfulfilled as all current methods have limitations: low transduction efficiency; stable maintenance in host cells without integration into the host genome; appropriate expression; no risk of cellular transformation or stimulation of the host's immune system.

An alternative solution to overcome all these problems could be the use

of human artificial chromosome (HAC) (Kazuki and Oshimura, 2011). HACs are exogenous mini-chromosomes that can work as gene delivery vectors when transduced into stem or progenitor cells through a technique called “microcell-mediated chromosome transfer” (MMCT) (Ren et al., 2006). HACs construction could be performed both by a “top-down approach” (engineered chromosomes; (Kuroiwa et al., 1998) or a “bottom-up approach” (de novo artificial chromosomes; (Harrington et al., 1997a). Although the efficiency of HACs transferring into target cells is lower than that of conventional viral vectors, they present a deal of advantages over other conventional gene delivery vectors: I) since they are episomal, they replicate and segregate as natural chromosomes independently from the host genome, thus minimizing the problems of insertional mutagenesis and silencing (Harrington et al., 1997a; Ikeno et al., 1998; Guiducci et al., 1999; Kuroiwa et al., 2000; Katoh et al., 2004); II) HACs can faithfully mimic the normal pattern of gene expression because they can accommodate entire genomic loci, including upstream and downstream regulatory elements (Tomizuka et al., 1997); III) it is possible to maintain long-term correction of mutated genes because these vectors are mitotically stable throughout many cell divisions in human cells (Bayne et al., 2004; Ren et al., 2005) (Fig 2.1.3.2.1).

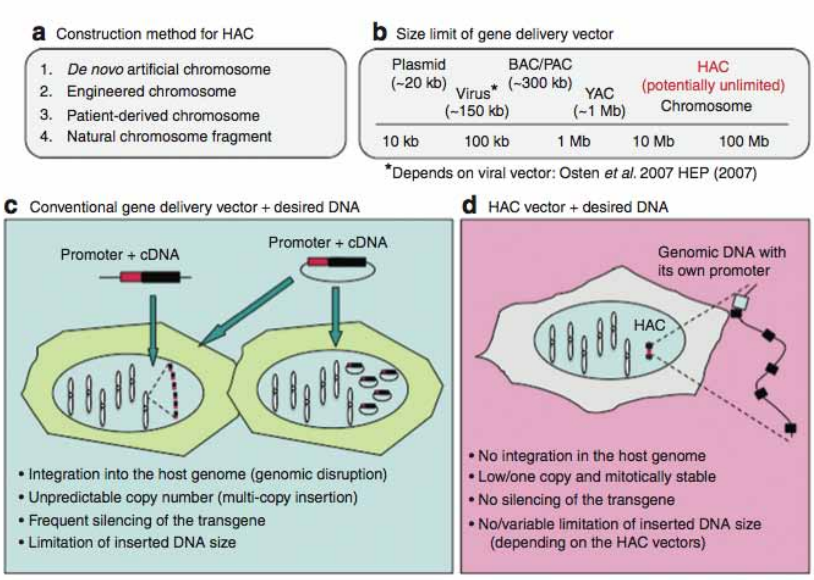


Figure 2.1.3.2.1 - Features of human artificial chromosomes (HACs).

(a) Different techniques to construct HACs. **(b)** Size limits of gene delivery vectors. Maximum deliverable DNA size in each vector is described. HAC vectors can carry DNA larger than 1 Mb. The size limits depend on each vector. **(c,d)** Limitations and consequences of gene delivery with conventional vectors such as a virus or plasmid, and with HACs, respectively. Taken from Kazuki and Oshimura, 2011.

A new HAC vector containing the entire human dystrophin locus (DYS-HAC) has been recently generated (Hoshiya et al., 2009) (Figure 2.1.3.2.2). This construct was transferred into mouse embryonic stem cells (ESCs) to create chimaeric pups expressing human dystrophin and could also be stably maintained into mesenchymal stem cells through their *in vitro* life. Most importantly, the same construct has been transferred to human DMD induced Pluripotent Stem Cells (iPSCs) (Kazuki et al., 2010), providing a unique gene and cell therapy tool for DMD. Surprisingly, so far there were no reports describing functional evidences of HAC efficacy in any animal model of genetic disease using stem cell-mediated gene-replacement therapy. Recently, the

transplantation into a murine model of DMD (*scid/mdx* mouse) of murine dystrophic mesoangioblasts (mdxMABs), previously corrected with the DYS-HAC, results into a clear morphological and functional amelioration of the pathological phenotype (Tedesco et al., 2011). This is the first evidence of efficacy of a stem cell and HAC-mediated gene therapy in a preclinical model of DMD raising the potential for future clinical translation.

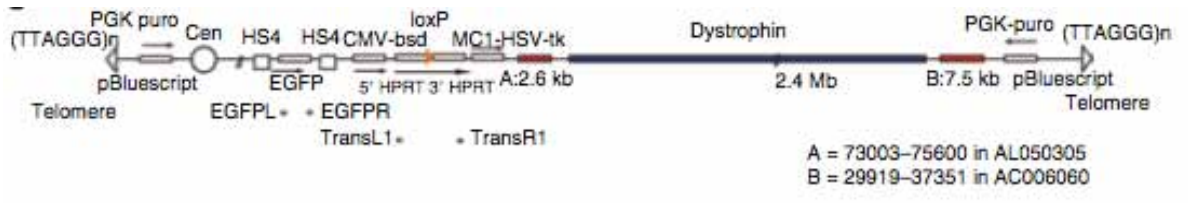


Figure 2.1.3.2.2 - Map of dystrophin human artificial chromosome (DYS-HAC).

The DYS-HAC vector contains the enhanced green fluorescent protein (EGFP) gene, the herpes simplex virus thymidine kinase (HSV-tk) gene, and several selection markers (bsd: blasticidin; puro: puromycin; HPRT gene). Both telomeres of the DYS-HAC are artificial. The centromere of the DYS-HAC is derived from human chromosome 21. Adapted from Hoshiya et al., 2009.

We can conclude this section by stating that potential application of HACs ranges from classical treatment of genetic disorders through gene and cell therapy by transferring the desired HAC into stem/progenitor/iPS cells, as well as generation of chimaeric mice to perform functional analyses *in vivo* (Fig. 2.1.3.2.3).

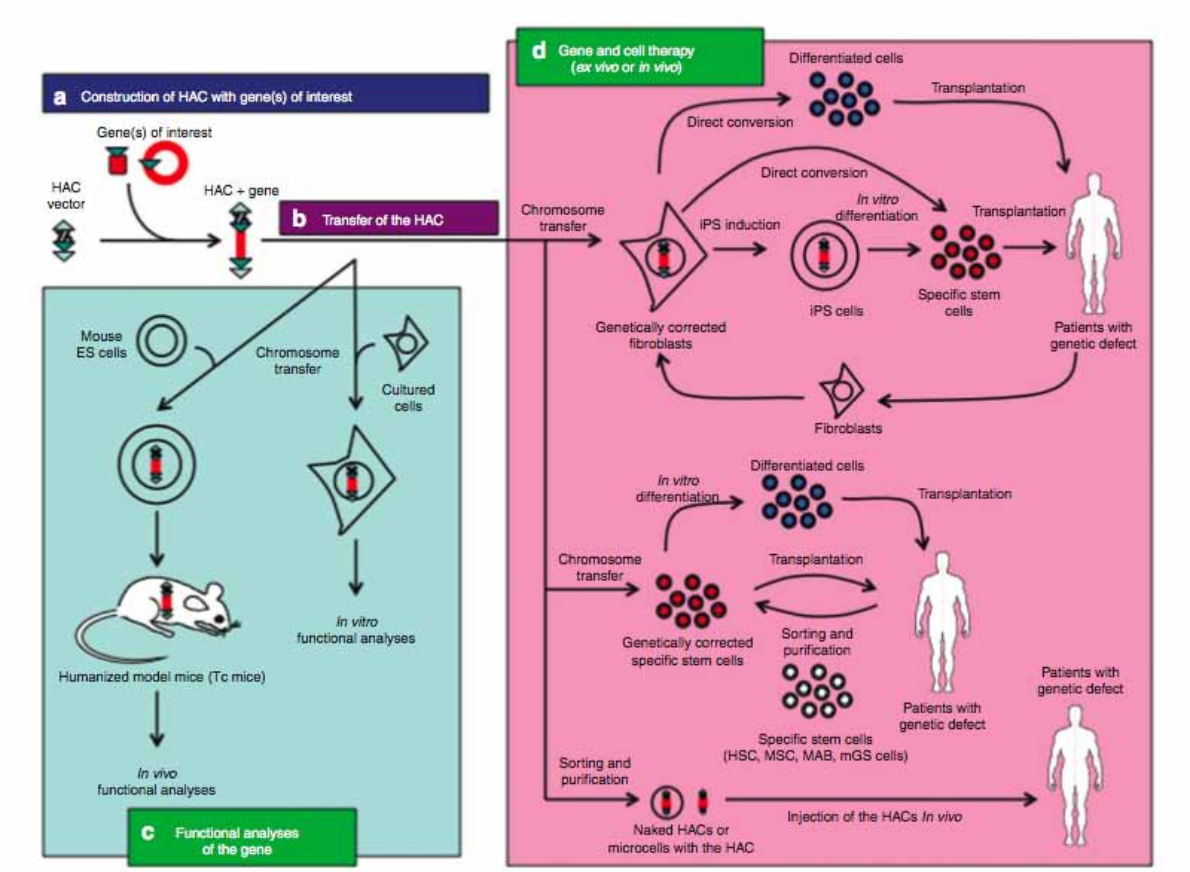


Figure 2.1.3.2.3 - Schematic diagram of HAC employment to the treatment of genetic disease and *in vivo* functional analysis. a) HAC construction with gene(s) of interest b) Microcell mediated transfer (MMCT) of HAC either into c) mouse ES cells in order to generate chimaeric mice and d) for the gene and cell therapy using as a recipient specific stem cells or iPS. HSC, hematopoietic stem cell; iPS, induced pluripotent stem; MAB, mesoangioblast; mGS, multipotent germline stem; MSC, mesenchymal stem cell. Taken from Kazuki and Oshimura, 2011.

2.1.3.3 Cell therapy

The possibility of restoring proper gene expression into skeletal muscle via allogeneic or autologous, genetically corrected stem cell transplantation is already possible thanks to a number of studies on skeletal muscle stem cells. However, the cell therapies approaches still remains very expensive and in need of proof of efficacy as all the other strategies. Nevertheless, stem cell-based therapies will likely be used together with drugs (Chinen and Buckley, 2010), so that the future personalized medicine for MDs will certainly integrate different classical (i.e. drugs) and innovative therapies (i.e. gene repair/replacement) in order to achieve disease- and patient-specific definitive cures.

In the last 80s, pioneering experiments in *mdx* mouse clearly showed that myoblasts could be transplanted into dystrophic muscle giving rise to dystrophin-expressing myofibers (Partridge et al., 1989). After this important discovery, different clinical trials for DMD started in the early 1990s; once finished, unfortunately they demonstrated the safety but also the absence of evident functional benefit derived from myoblast injections into the dystrophic injected muscles. This was mainly due to the poor survival and migration of myoblasts (Mouly et al., 2005). Subsequent experiments have been focused to optimize the use of myoblasts to treat MDs and a Phase I clinical trial has been completed some years ago (Skuk et al., 2006). Although encouraging results have been obtained, local administration is still a strong limiting point.

In the last ten years, several adult-derived stem cells have been isolated (Jiang et al., 2002) (see 2.2.2 **Unconventional myogenic stem cells**); they have been characterized and used in animal transplantation experiments, and the promising results have opened up new possibilities for cell therapy in

muscular dystrophies (Peault et al., 2007).

2.1.3.4 Mutation-specific strategies

In this last years, other two interesting therapeutic approaches with the aim of targeting dystrophin genetic defect, have been developed: exon-skipping and nonsense codon suppression. The final goal is to target mRNA splicing or termination with these small molecules respectively (Nelson et al., 2009) and both strategies are now in phase II/III clinical trials (van Deutekom et al., 2007; Welch et al., 2007; Kinali et al., 2009). As a result, a Duchenne patient could turn into the milder Becker phenotype, since the action mechanism of exon-skipping or nonsense codon suppression lead to the expression of smaller but partially or completely functional dystrophin protein. Actually not all the mutations are "skippable" (Aartsma-Rus et al., 2009), depending where the mutation is located onto the gene, but is clearly known that the majority of dystrophin mutations are deletions within rod domain (from exon 44 to exon 55), which cause an alterated reading frame of dystrophin and result in a prematurely truncated protein (Muntoni et al., 2003).

In this direction, exon-skipping has been tested in dystrophic animal models (Muntoni et al., 2003) and DMD trials (van Deutekom et al., 2007; Kinali et al., 2009). These studies utilize antisense oligonucleotides (AONs) and recently a variety of chemical variants and delivery methods have been tested. Two types of AONs are mainly used: 2′-O-methyl- phosphorothioate (2OMP) and phosphorodiamidate morpholino oligomer (PMO). Recently, a DMD trial based upon systemic PMO treatment showed the safety of exon-skipping strategy together with dystrophin expression restoration but no beneficial effects have

been shown (Cirak et al., 2011). Even if successful, exon-skipping has two major limitations: it requires life-long administration of very expensive oligonucleotides and is not applicable to all mutations. Another approach to systemically deliver antisense-based exon-skipping is through viral vectors which carry the cDNA of a U7 small nuclear RNA, a sequence complementary to the acceptor and donor splice site(s) of the exon(s) to be skipped (Goyenvalle et al., 2009). This strategy requires a gene therapy approach but, in contrast, it offers the possibility of permanent repair.

About the nonsense codon suppression strategy, roughly 10% of all dystrophin mutations lead to the creation of a nonsense stop codon that result in a truncated protein that is rapidly degraded. Aminoglycoside antibiotics have the capacity to reduce ribosomal fidelity for recognizing these premature termination codons in the dystrophin transcript and, through this mechanism, induce ribosomal read-through of premature termination signals with generation of a full-length protein with only one amino acid substitution (Barton-Davis et al., 1999). Aminoglycoside antibiotics were quite inefficient and showed significant toxicity after long-term administration. An extensive screening then led to a compound called PTC124 (ataluren) and phase I and II clinical trials demonstrated good safety and tolerability in DMD (Welch et al., 2007). Unfortunately, preliminary results of a large phase IIb clinical trial show that the functional amelioration caused by the molecule in treated patients did not reach statistical significance in comparison with controls.

2.2 SKELETAL MUSCLE STEM CELLS

2.2.1 Conventional myogenic stem cells: Satellite cells

2.2.1.1 Satellite cells origin and characterization

Skeletal muscle, the most abundant tissue of the body, has the ability to regenerate new muscle fibers after injury or as a consequence of diseases such as muscular dystrophy (Carlson, 1973). Muscle fibers contain several hundred nuclei within a continuous cytoplasm, therefore, whether the process of regeneration depends upon the fusion of mononucleated precursor cells or upon the fragmentation of dying muscle fibers, which release new cells, remained controversial for a long time, even after the demonstration by Beatrice Mintz and Wilber Baker that multinucleated fibers are formed by the fusion of single cells (Mintz and Baker, 1967). Thanks to the pioneering work of Alexander Mauro we know that, during muscle regeneration, the main role is played by satellite cells (SCs), the canonical and resident stem cells of skeletal muscle (Mauro, 1961).

SCs have been described for the first time as mononuclear cells located between the basal lamina of skeletal fibers, closely juxtaposed to the plasma membrane (Mauro, 1961) (Figure 2.2.1.1 A). SCs originate from somites (Shi and Garry, 2006; Sambasivan and Tajbakhsh, 2007), which are spheres of paraxial mesoderm that will generate skeletal muscle, dermis, and axial skeleton, but is still unknown the exact progenitor that gives rise to SCs.

In physiological condition, SCs are present in healthy adult mammalian muscle as quiescent cells representing approximately the 2.5–6% of all nuclei of a muscle fiber (Beauchamp et al., 2000). In their quiescent state they expressed a

panel of characteristic, but not unique, markers (summarized in Table 2.2.1.1): Pax7 (Zammit et al. 2006), Myf5 (Tajbakhsh et al., 1996), CD34 (Beauchamp et al., 2000), M-cadherin (Irintchev et al., 1994), VCAM-1 (Rosen et al., 1992), c-met (HGF, hepatocyte growth factor, receptor) (Cornelison and Wold, 1997), CAM-1 (cell adhesion molecule-1) (Bischoff and Heintz, 1994), foxk1 (Garry et al., 2000) and Sydecans3 and 4 (Cornelison, 2001). In particular, the most important and widely used marker is Pax7, a transcriptional factor essential for SCs specification and survival (Kuang et al., 2006). In contrast, Pax3 is expressed only in quiescent SCs in a few specific muscle groups such as the diaphragm (Relaix et al., 2006). The basic helix-loop-helix (bHLH) myogenic regulatory factor 5 (Myf5) gene is expressed in the large majority of quiescent SCs, and for this reason mice expressing nuclear LacZ under the control of the Myf5 promoter (*Myf5*$^{nlacZ/+}$ mice) have been useful for identifying and characterizing SCs (Tajbakhsh et al., 1996) (Figure 2.2.1.1 B). Some of the other listened surface markers are used by cell sorting for isolating “purified” SC populations, but since each marker is not exclusively expressed on SCs, a combination of different markers must be used. Alternatively, transgenic mice such as those expressing GFP under promoters that drive the expression of genes encoding SC markers, such as the Pax3 promoter, can be used to isolate SCs (Montarras et al., 2005; Day et al., 2007; Bosnakovski et al., 2008).

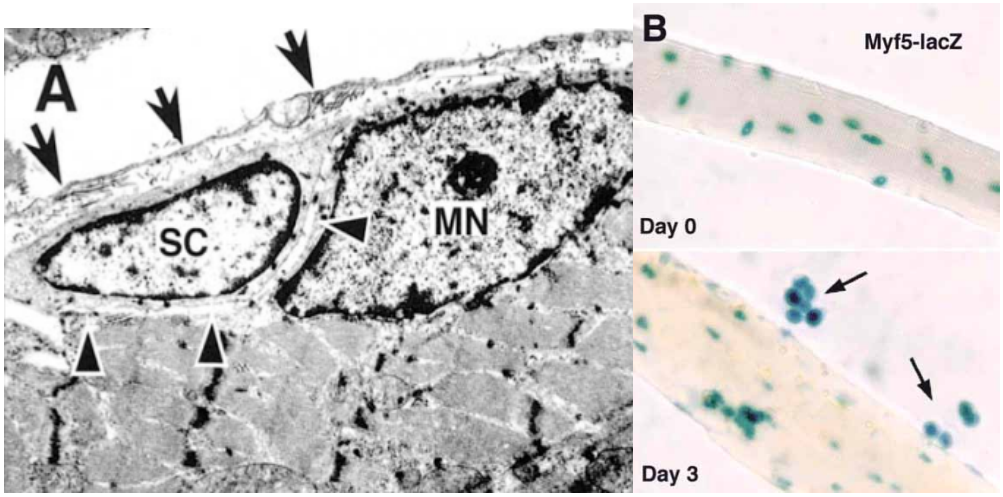


Figure 2.2.1.1 - Satellite Cell. (A) Transmission electron micrograph of a satellite cell (SC) as identified by the plasma membrane (arrowheads) separating the satellite cell from adjacent myofibers, the continuous basal lamina surrounding the satellite cell and myofibers (arrows), and the heterochromatic appearance of the nucleus. MN, myonucleus within fiber nuclei. **(B)** Myf5-nlacZ is expressed in myonuclei in fresh (day 0) single muscle fibers as well as activated satellite cells and myoblasts (day3) derived from Myf5-nlacZ mice (Beauchamp et al., 2000). Adapetd from Seale et al., 2001 (Seale et al., 2001).

In humans, SCs markers do not fully correspond to those of the mouse. As an example, human SCs are CD34 and M-cadherin negative (Peault et al., 2007) whereas the most common marker is CD56, although it also marks natural killer (NK) lymphocytes (Illa et al., 1992).

Table 2.2.1.1 - Satellite cell markers.

Markers (ref.)	**SC expression**	**Localization and function**	**Prospective isolation***	**Expression in other tissues/cells**
Pax7 (Zammit et al., 2006)	100% of quiescent and activated SCs	Nuclear; Transcription factor	Pax7-GFP mouse	Absent
Pax3 (Relaix et al., 2006)	Quiescent SCs (only in a subset of muscles)	Nuclear; Transcription factor	Pax3-GFP mouse	Melanocyte stem cells, Shwann cells
Myf5 (Tajbakhsh et al., 1996)	Most quiescent SCs and all proliferating SCs and myoblasts	Nuclear; Transcription factor	Myf5-nLacZ	Absent
Syndecan3 and 4 (Cornelison et al., 2001)	98% of quiescent and activated SCs	Membrane; Transmembrane heparan sulfate proteoglycan	Cell sorting	Brain, dermis, bone marrow, bone, smooth muscle, tumours
VCAM-1 (Rosen et al., 1992)	Quiescent and activated SCs	Membrane; Adhesion molecule	Cell sorting	Activated Endothelial cells
c-met (Cornelison and Wold, 1997)	Quiescent and activated SCs	Membrane; HGF receptor	Not used	Many tissue and tumours
Foxk1 (Garry et al., 2000)	Quiescent and activated SCs	Nuclear factor	Not used	Neurons
Cd34 (Beauchamp et al., 2000)	Quiescent and activated SCs	Membrane protein	Cells sorting	Hematopoietic, endothelial, mast and dendritic cells
M-cadherin (Irintchev et al., 1994)	Quiescent and activated SCs; Myoblasts	Membrane; Adhesion protein	Not used	Absent
Caveolin-1 (Gnocchi et al., 2009)	Quiescent and activated SCs; Myoblasts	Membrane protein	Not used	Endothelial fibrous and adipose tissue
Integrin α7 (Blanco-Bose et al., 2001)	Quiescent and activated SCs; Myoblasts	Membrane; Adhesion protein	Cells sorting	Vessel associated cells
Integrin β1 (Kuang et al., 2007)	Quiescent and activated SCs	Membrane; adhesion protein	Cell sorting	Many tissues
Cd56 (Betsholtz, 2004)	Quiescent and activated SCs; Myoblasts	Membrane; homophilic binding glycoprotein	Cell Sorting	Glia, neurons and natural killer cells
SM/C2.6 (Fukada et al., 2004)	Quiescent and activated SCs; Myoblasts	Unknown	Cell sorting	Unknown
Cxcr4 (Sherwood et al., 2004)	Subset of quiescent SCs	Membrane; SDF1 receptor	Cell sorting	Hematopoietic stem cells, vascular endothelial cells and neuronal cells
Nestin (Day et al., 2007)	Around 98% of quiescent SCs and myoblasts	Intermediate filament protein	Nestin GFP mouse	Neuronal precursors cells

* Prospective isolation: Direct isolation of cells from tissue, usually based upon cyto-fluorimetric sorting with antibodies directed against cell surface markers. Adapted from Tedesco et al., 2010.

2.2.1.2 Satellite cells activation

As previously described above, SCs are in a quiescent state during the major part of their life. In response to a muscle injury, SCs turn into an activate state starting to proliferate; at this point they are called myoblasts or myogenic precursor cells (mpc) (Dhawan and Rando, 2005; Price et al., 2007). There are several signals involved in SC activation, including HGF (Tatsumi et al., 1998), FGF (Floss et al., 1997), IGF (Musaro, 2005) and NO (Wozniak and Anderson, 2007), which come both from damaged skeletal muscle fibers and inflammatory cells.

Activated SCs myogenic differentiation is mainly driven by Myf5 and MyoD (Tajbakhsh et al., 1996) and is followed by fusion into regenerating fibers. In the mouse, the process through which activated SCs fuse together to form skeletal muscle fibers takes roughly seven days (Zammit et al., 2002). During this time every single SC need to choose between different fates: the majority turn into $Pax7^{+}MyoD^{+}$ cells, which are committed to differentiation (Zammit et al., 2004) whereas few SC downregulate MyoD and return to quiescence (to maintain the progenitor pool). This mechanism, through which SCs undergo asymmetric division maintaining self-renewal and a pool of progenitor ready to use, is finely controlled but is still unclear which molecules are involved in; among these, Notch signalling pathway is thought to regulate this process by promoting asymmetric divisions, although there is not agreement on the role of Numb (a Notch inhibitor and a cell-fate determinant) in inducing differentiation (Conboy and Rando, 2002) and sustaining self-renewal (Shinin et al., 2006).

The asymmetric cell division is also demonstrated by the identification of a sub-population of SCs able to retain BrdU after pulse-chase labelling, with some cells

displaying selective template DNA strand segregation during mitosis (Shinin et al., 2006; Conboy et al., 2007). Moreover, Rudnick's team confirmed the label-retention model of SCs and demonstrated that approximately 10% of $Pax7^+$ mouse SCs had never expressed Myf5 and that these cells are adherent to the basal lamina during asymmetric mitosis, generating one $Pax7^+Myf5^-$ satellite "stem cell" and one $Pax7^+Myf5^+$ SC "progenitor", eventually fated to differentiate (Kuang et al., 2007) (Figure 2.2.1.2.). The same group also described that Wnt7a regulates the symmetric expansion of $Pax7^+Myf5^-$ SCs (Le Grand et al., 2009).

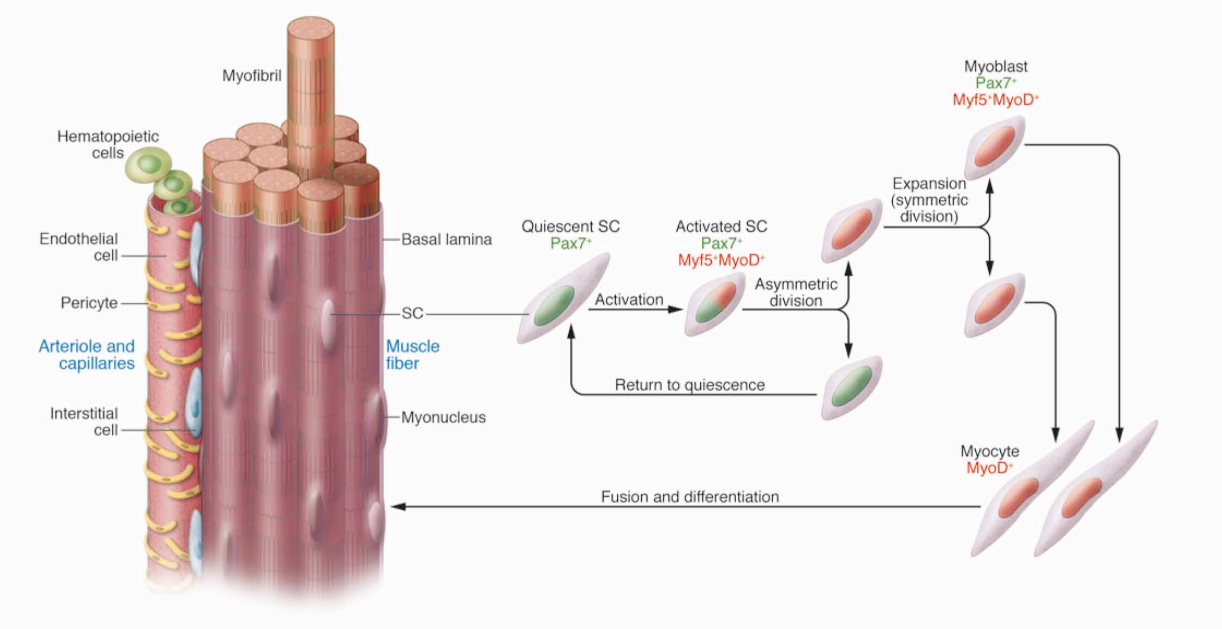


Figure 2.2.1.2 - Asymmetric cell division during activation of SCs.

This figure illustrates the anatomy of a muscle fiber together with an adjacent small vessel. SCs and other unconventional myogenic cells (pericytes and hematopoietic, endothelial, and interstitial cells) are also depicted. SC activation *in vivo* is followed by an asymmetric division, with Pax7, MyoD, and Myf5 being expressed in differentiating cells and Pax7 in cells returning to quiescence in order to maintain a pool of progenitors. Adapted from Tedesco et al., 2010.

2.2.2 Unconventional myogenic progenitors

The availability of cell-autonomous tissue-specific transgenic markers, allowed the unequivocal demonstration of the existence of myogenic progenitors originating from tissues other than skeletal muscle (Cossu, 1997). Upon transplantation (either bone marrow transplantation (BMT) or direct injection into skeletal muscle), these cells identified by transgene expression, participate in muscle regeneration of wild type and/or dystrophic mice and eventually enter the SC pool. The possibility that myogenic differentiation may depend upon fusion (and hence exposure to the dominant activity of MyoD) remains but, for skeletal muscle, this would be part of the physiological mechanism that creates the tissue. Below some examples of these unorthodox myogenic cells have been briefly described.

2.2.2.1 Cells derived from ectoderm: neural stem cells

To date, both murine and human neural stem cells are the only ectoderm-derived stem cells that have been shown to differentiate into skeletal muscle when co-cultured with skeletal myoblasts or transplanted into regenerating skeletal muscle (Galli et al., 2000). Interestingly, cells expressing Myf5 exist in the brain and spinal cord, suggesting a cryptic potency that becomes apparent *in vitro* (Tajbakhsh et al., 1994).

2.2.2.2 Hematopoietic cells (HSCs)

The first evidence of *in vivo* generation of skeletal muscle from bone marrow cells was reported in 1998**,** in a study that used transgenic mice expressing a nuclear LacZ under the control of the striated muscle promoter *myosin light*

chain 1/3 fast (MLC3f) (Ferrari et al., 1998). After bone marrow transplantation (BMT) from the transgenic mice to an injured host muscle, unequivocal β-gal positive nuclei were detected in regenerated fibers, demonstrating that murine bone marrow contains transplantable progenitors that can be recruited through the circulation to an injured muscle, where they participate in muscle repair (Ferrari et al., 1998). This opened the possibility of treating muscular dystrophy by BMT, but work in mice indicated that, unfortunately, the frequency of this event was too low, even in a chronically regenerating dystrophic muscle and even if the SP (side population) progenitor–enriched fraction was transplanted (Gussoni et al., 1999; Ferrari et al., 2001).

To address this issue, subsequent experiments were directed to identifying a rare, potentially highly myogenic progenitor, but those studies have so far had modest success. The hematopoietic $CD45^+$ fraction of the bone marrow has been identified as the cell population with myogenic potential (McKinney-Freeman et al., 2002) and retrospective analysis in a DMD patient that had undergone BMT confirmed the persistence of donor-derived skeletal muscle cells over a period of many years, again at very low frequency (Gussoni et al., 2002). Together these data suggested that HSCs, or a yet to be identified cell that expresses several markers in common with true HSCs, has myogenic potential. More recent approaches confirmed that hematopoietic cells have myogenic potential but disagreed on the stage at which myogenic differentiation would occur. One study reported that the progeny of a single mouse hematopoietic progenitor cell can both reconstitute the hematopoietic system and contribute, at low frequency, to muscle regeneration (Corbel et al., 2003). However, a similar study showed that in response to injury, $CD45^+$ hematopoietic

progenitors contribute to regenerating mouse skeletal muscle through fusion of mature myeloid cells rather than fusion of the HSCs (Camargo et al., 2003).

A subpopulation of circulating cells expressing CD133 (also known as AC133), a well-characterized marker of HSCs, also expresses early myogenic markers (Torrente et al., 2004). When injected into the circulation of dystrophic immunodeficient *scid/mdx* mice, $CD133^+$ cells have been found to contribute to muscle repair, recovery of force, and replenishment of the SC pool. The same group also isolated a population of muscle-derived stem cells expressing CD133 (Benchaouir et al., 2007). Furthermore, when $CD133^+$ cells from DMD patients were genetically corrected by lentivirus-mediated exon-skipping for dystrophin exon 51, they were able to mediate morphological and functional recovery in *scid/mdx* mice (Benchaouir et al., 2007). Thus, different sub-populations of hematopoietic cells, whose characterization is still incomplete, seem to possess myogenic potency, but none of these exhibit this property at high frequency.

2.2.2.3 Cells derived from the mesoderm

Many different types of mesoderm stem/progenitor cells have been shown to exhibit myogenic potential, usually after drug treatment, genetic modification, or co-culture with SCs or myoblasts. In some cases evidence of *in vivo* myogenesis has been documented. The list of such cells includes mesenchymal stem cells (MSCs), multipotent adult progenitor cells (MAPCs), muscle-derived stem cells (MDSCs), $CD133^+$ cells, mesoangioblasts (MABs, see in details section 1.3 Mesoangioblasts), endothelial progenitor cells (EPCs), and adipose-derived stem cells. More details can be found in previous reviews (Cossu and Sampaolesi, 2007; Peault et al., 2007).

MSCs have been shown to be capable of skeletal myogenesis (Dezawa et al., 2005). However, recently, Perlingeiro and colleagues demonstrated that although Pax3 activation enabled the *in vitro* differentiation of murine and human MSCs into $MyoD^+$ myogenic cells, these cells failed to cause functional muscle recovery in *mdx* mice, despite good engraftment (Gang et al., 2009). The reason for this failure remains unclear.

Initially identified as circulating cells expressing CD34 and fetal liver kinase-1 (Flk-1; also known as VEGFR2), EPCs (Asahara et al., 1997) were shown to be transplantable and to participate actively in angiogenesis in various physiologic and pathologic conditions (Asahara et al., 1997). It was then shown that freshly isolated human cord blood $CD34^+$ cells injected into ischemic adductor muscles gave rise not only to endothelial but also to skeletal muscle cells in mice (Pesce et al., 2003). Consistent with this, Peault and colleagues have identified cells with high myogenic potential within the vascular endothelium of human adult skeletal muscle (Zheng et al., 2007). These human myoendothelial cells, which represented less than 0.5% of the cells in dissociated adult skeletal muscles, expressed both myogenic and endothelial cell markers ($CD56^+$, $CD34^+$, $CD144^+$, $CD45^-$), exhibited long term proliferation, had a normal karyotype, and when transplanted into *scid* mice were able to regenerate fibers in injured muscle (Zheng et al., 2007).

Human multipotent adipose-derived stem (hMADS) cells, isolated from adipose tissue, differentiate into adipocytes, osteoblasts, and myoblasts (Meliga et al., 2007). Recently, the myogenic and muscle repair capacities of hMADS have been enhanced by transient expression of MyoD (Goudenege et al., 2009). The easy availability of their tissue source, their capacity for expansion *ex vivo*,

their multipotent differentiation, and their immune-privileged behaviour, suggest that hMADS cells could be an important tool for cell-mediated therapy for skeletal muscle disorders, although more robust functional evidences are needed.

2.2.3 Skeletal muscle stem cells: preclinical DMD models and clinical trials

Because of their features, SCs in particular were considered the best candidate for a cell therapy approach to treat muscular dystrophy. Pioneer studies demonstrated that intramuscular injection of normal myoblasts (Partridge et al., 1989) into *mdx* mice, which lack dystrophin and are a model for DMD, resulted in fusion with host fibers and extensive dystrophin production.

As a consequence of this brilliant results obtained in mice, the early 1990s reported the first myoblast transplantation in a young DMD boy demonstrating myoblast injections safety as well as dystrophin production (Law et al., 1990). Based upon these evidences, a great number of clinical trials were performed in DMD patients failing for a number of reasons (Law et al., 1991; Gussoni et al., 1992; Huard et al., 1992; Law et al., 1992; Karpati et al., 1993; Tremblay et al., 1993; Mendell et al., 1995; Morandi et al., 1995; Miller et al., 1997; Neumeyer et al., 1998; Skuk et al., 2006). As a matter of fact, all these completely independent clinical experimentations testified the absence of any adverse events; otherwise not all showed dystrophin production and, in addition, no one can affirmed that there were some benefits from these injections (Partridge, 2000; Cossu and Sampaolesi, 2007).

Probably this was due to different problems: I) intramuscular injection in several locations of a single muscle (or at most a few muscles) cannot elicit a general

effect and so II) intramuscularly injected cells distribute locally, implying that a huge number of injections will have to be performed in order to treat a complete muscle (Huard et al., 1992); III) immune responses toward the injected SCs; IV) the quick death of the majority of the myoblasts in the first 72hrs after injection (Fan et al., 1996; Guerette et al., 1997).

Many subsequent preclinical studies aimed to improve the survival, proliferation, and differentiation of the SCs after engraftment. For example, transplantation in dystrophic mouse muscles of a single muscle fiber, which contained as few as seven SCs, led to an increasing number of new SCs that in turn generated more than one hundred new muscle fibers and could also be activated after injury (Collins et al., 2005). This is a much more efficient way to generate new muscle fibers than transplantation of cultured SCs, where normally the number of donor-derived new fibers that are generated is several orders of magnitude less than the number of injected cells. Unfortunately, this method would be difficult to translate into clinical protocols. In addition, in the past few years several groups have succeeded in prospectively isolating “pure” populations of SCs by using a combination of different markers (Montarras et al., 2005). All these studies revealed that freshly isolated cells have a much greater capacity to generate dystrophin-expressing fibers in *mdx* mice than the same cells after *in vitro* expansion (Montarras et al., 2005).

Recently, Torrente and colleagues reported the first $CD133^+$ cell transplant (Torrente et al., 2007). They designed a phase I double blind trial with an autologous transplant of unmodified, and thus still dystrophic, muscle-derived $CD133^+$ cells in 8 boys affected by DMD exclusively to test safety; and indeed,

no adverse events were reported. Another Phase I/II clinical trial on DMD patients is ongoing using mesoangioblasts.

2.3 MESOANGIOBLASTS

2.3.1 Origin and characterization of Mesoangioblasts

In the last 10 years, a variety of studies showed the existence in almost all the body tissues of progenitors able to differentiate with a different extent into several cell types that do not correlate with their embryological origin: as an example, as already mentioned bone marrow cells differentiate into skeletal muscle (Ferrari et al., 1998; Gussoni et al., 1999), liver (Lagasse et al., 2000) and Central Nervous System (CNS) (Kopen et al., 1999; Brazelton et al., 2000; Mezey and Chandross, 2000) whereas in the CNS there are cells with the ability to differentiate into hematopoietic stem cells or skeletal muscle (Galli et al., 2000).

All these findings completely changed the idea of an unperturbed cell lineage, introducing the concept of plasticity and stimulating a deal of studies with the final goal to discover the best cell able to generate the desirable tissue.

In this scenario, De Angelis and colleagues isolated from the embryonic murine dorsal aorta (a non canonical source of myogenic cells) a new subpopulation of cells expressing early endothelial markers but able to give rise to skeletal muscle (Bianco and Cossu, 1999; De Angelis et al., 1999; Minasi et al., 2002) (Figure 2.3.1.1). These cells have been called mesoangioblasts (MABs) to underline the

possibility of a common progenitor both for vascular and mesoderm extra-vascular tissues (Cossu and Bianco, 2003).

(Figure 2.3.1.2); this hypothesis was also supported by two distinct studies which suggested a common ancestor for endothelial cells and skeletal muscle fibers in somites (Kardon et al., 2002) and the existence of a common Flk1+ progenitor both for skeletal and cardiac muscle (Motoike et al., 2003).

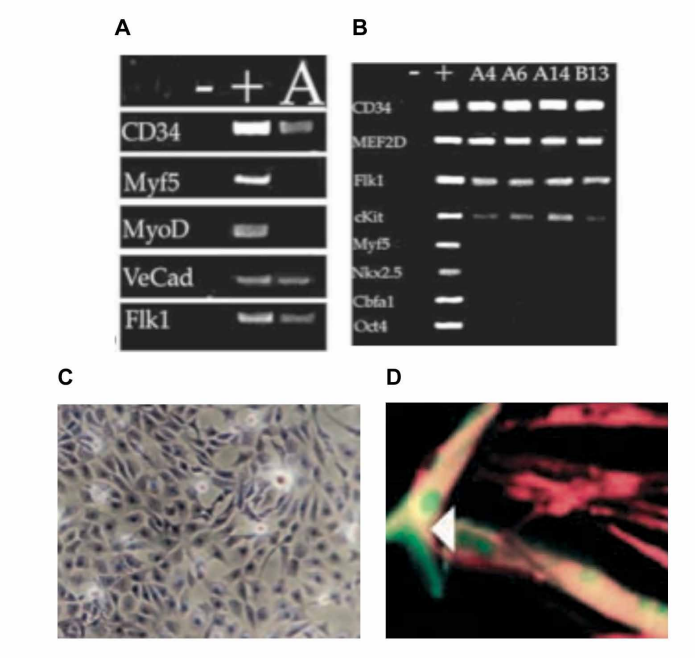


Figure 2.3.1.1 - Characterization of murine embryonic MABs isolated from dorsal aorta of E9.5 mouse embryos. A) RT-PCR panel revealed that endothelial and hematopoietic markers (VE-Cad, Flk1 and CD34), but not myogenic markers (Myf5 and MyoD), were expressed in dissected aorta (A). Total embryo extract was used as a positive control (+). Negative control (no RNA) is shown in the first lane (–). **B)** RT-PCR of the messages expressed by several cell lines (A4, A6, A14 and B13) from the dorsal aorta after 5 passages *in vitro*. Note expression of hemo-angioblastic but not of tissue specific markers such as Myf5 or Nkx2.5. **C)** Phase contrast morphology of one typical clone from embryonic aorta after passages *in vitro*. **D)** Skeletal myotubes are detected after co-culture of GFP-labelled MABs with C2C12 myoblasts. GFP-

positive cells appear green, myocytes and myotubes expressing myosin heavy chains appear red, and cells expressing both appear yellow in the merged image (arrowhead). Adapted from Minasi et al. 2002.

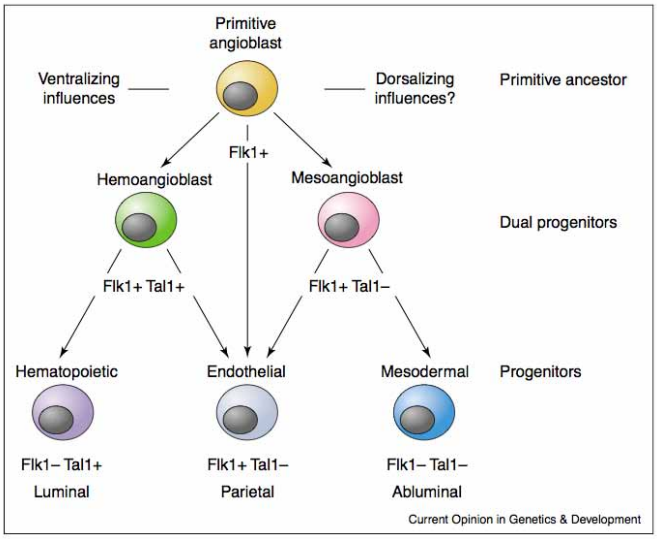


Figure 2.3.1.2 - A hypothetical scheme trying to define the origin of the mesoangioblast. Environmental (ventral) cues drive a subset of angiopoietic progenitors to a hematopoietic fate. Unknown cues preserve a potential for differentiation towards mesodermal lineage to another subset of angiopoietic progenitors. This potential is retained in the offspring of the Flk-1þ ancestor, defining a Flk-1 multiple mesodermal progenitor. Asymmetric divisions segregate the hematopoietic progeny of the hemangioblast in the luminal space, the progeny of mesoangioblasts in the subendothelial, abluminal space. Taken from Cossu and Bianco, 2003.

As a matter of fact, murine MABs express Flk1 and other early but not late endothelial markers; due to their vascular origin, they are able to cross the vessel wall and, since it is easily possible to transduce them with lentiviral vectors, they have been used in preclinical models for muscular dystrophies. As result, intra-arterial delivery of either wild type or genetically corrected MABs ameliorated the dystrophic phenotype of mice lacking α-sarcoglycan, a model for LGMD (Sampaolesi et al., 2003). In addition, intra-arterial delivery of autologous adult canine MABs resulted too in extensive recovery of dystrophin expression

and ameliorated pathologic muscle morphology and function in Golden Retriever dystrophic dogs (GRMD), the canine model for DMD (Sampaolesi et al., 2006).

Cells with a similar features and behaviour have been isolated from microvasculature of human post-natal skeletal muscle (Dellavalle et al., 2007); these interstitial cells seem to represent a subset of pericytes since they expressed markers such as annexin, vimentin, desmin, PDGF receptor β (Platelet-Derived Growth Factor beta) SMA (Smooth Alpha Actin) (Armulik et al., 2005), NG2 proteoglycan and, most importantly, they are positive for Alkaline Phosphatase (ALP), an exclusive marker of perycites in skeletal muscle (Safadi et al., 1991). On the other hand, in contrast with embryonic murine MABs, human cells do not express any endothelial markers.

These findings suggested that vessel-associated ALP+ human interstitial cells obtained from post-natal muscle biopsies could be considered the progeny of pre-natal mesoangioblasts that, during progression from a pre-natal to an adult state, switched from an endothelial-like to a pericyte-like phenotype. For this reason we called them pericyte-derived cells and they could considered the human counterpart (human MABs) of embryonic murine MABs. *In vivo*, when transplanted intra-arterially into *scid/mdx* mice, human MABs give rise to dystrophin-positive muscle fibers ameliorating function and morphology of the dystrophic phenotype (Dellavalle et al. 2007).

These results supported the evidence that human MAB (or pericyte-derived cell) represent a second myogenic precursor resident in adult human skeletal muscle but clearly distinct from a satellite cell; moreover, MABs are able to generate skeletal fibers expressing dystrophin when transplanted into a dystrophic muscle.

As mentioned above, MABs are distinct from satellite cells, the canonical and resident myogenic stem cells, for a number of fundamental differences. Firstly, they have an *in vivo* different adjacent anatomical position; pericytes are located underneath the basal lamina of small vessels, whereas satellite cells are located inside the basal lamina of muscle fibers. Secondly, satellite cells express MyoD, Pax7, Myf5, MEF2C, CD56 and M-cadherin, which are not expressed in human MABs that rather express ALP (not expressed in satellite cells) and other markers such as SMA, PDGFRb and desmin, that however are also expressed by activated satellite cells. Moreover human MABs express MyoD and Myf5 only on terminal differentiation, suggesting distinct myogenic differentiation kinetics (Fig. 2.3.1.3). Third and most important difference is related to the vascular origin of pericyte-derived cells, which confers the ability to cross of the vessel walls, a feature that is absent in satellite cell-derived myoblasts.

Recently, we demonstrated that in mice pericytes transgenically labelled with an inducible ALP (Alkaline Phosphatase) CreERT2 are able to fuse with developing skeletal fibers and enter the satellite cell pool during normal post-natal development. Moreover, the contribution of pericytes to muscle fibers increases threefold after acute injury or during chronic regeneration that occurs in muscular dystrophy (Dellavalle et al., 2011). Hence, we can conclude that MABs contribution to skeletal muscle happens during physiological post-natal development and is enhanced in pathological conditions. Taken together, these findings strongly support the idea that MABs could be considered as an ideal cell population for the future cell/gene therapy of muscular dystrophies.

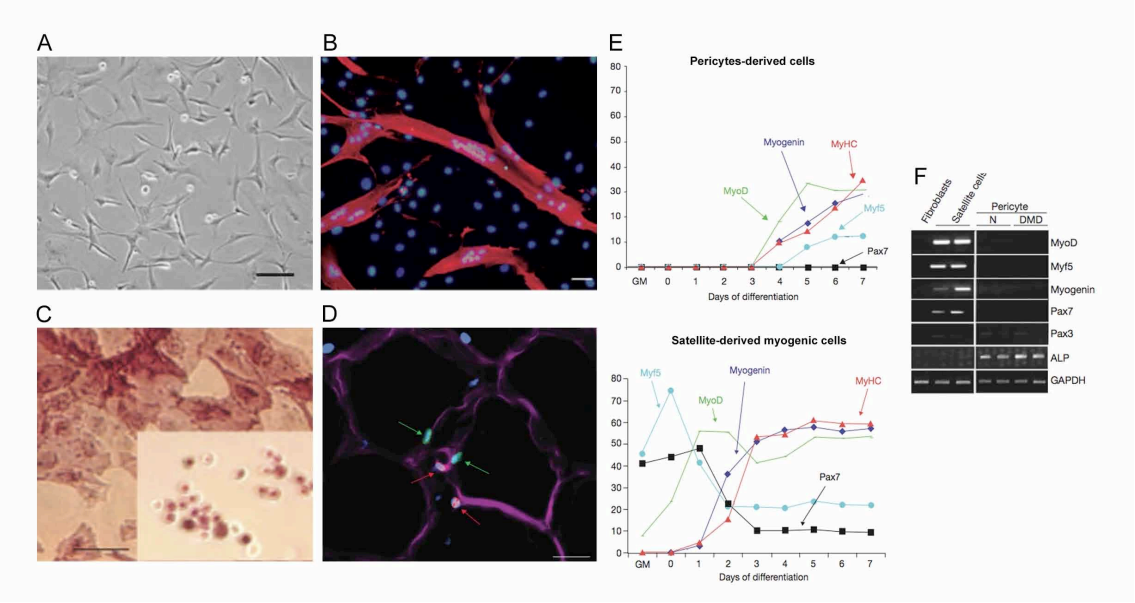


Figure 2.3.1.3 - *In vitro* characterization of human MABs and comparison with human satellite-derived cells. A) Phase-contrast morphology of a polyclonal population isolated from an explant culture after 5 passages in vitro. **B)** Spontaneous differentiation of human pericyte-derived cells, cultured in differentiation medium on matrigel coated dishes. **C)** Staining for ALP indicates expression at varying levels in most of cells outgrown from muscle explants. The inset shows floating cells, just removed from the muscle explant, all of which also express ALP. **D)** Immunofluorescence microscopy of human normal muscle stained with antibodies against M-cadherin recognizing satellite cells (green arrows), laminin (magenta) and ALP (red) recognizing pericytes (red arrows). Nuclei are stained in blue with DAPI. E) The time-course of expression of different myogenic markers is shown for pericyte-derived cells (upper panel) and for satellite cells (lower panel). **F)** RT–PCR analysis of the expression of MyoD, Myf5, Myogenin, Pax7, Pax3 and ALP in human fibroblasts, satellite cells, normal (N) and DMD pericyte-derived cells. Scale bars are 10 μm in A), 25 μm in B and 20 μm in C), D). Adapted from Dellavalle et al., 2007.

2.3.2 Cell and gene therapy: past, present and future

As described above, MABs are vessel-associated progenitors that express endothelial markers when isolated from the embryo or perycite markers when isolated from post-natal tissue. Thanks to their ability to differentiate into skeletal

muscle and to cross vessel wall. They can be delivered through the arterial circulation, reaching large areas of skeletal muscle tissue. Since MABs fulfil all the criteria required for a cell type to be used for gene and cell therapy (they are isolable from adult muscle biopsies, can be expanded *in vitro*, can be transduced with viral vectors and, most importantly), MABs have been used in preclinical models of DMD. Intra-arterial delivery of either wild type or genetically corrected murine MABs morphologically and functionally ameliorated the dystrophic phenotype of mice lacking α-sarcoglycan (Sampaolesi et al., 2003) (Figure 2.3.2.1). In addition, intra-arterial delivery of wild-type post-natal canine MABs resulted in extensive recovery of dystrophin expression and ameliorated pathologic muscle morphology and function in GRMD dogs (Sampaolesi et al., 2006) (Figure 2.3.2.2).

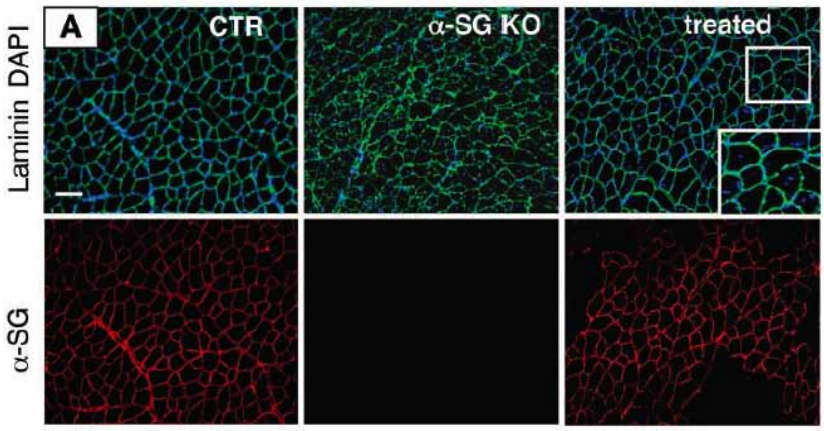


Figure 2.3.2.1 - Alpha-sarcoglycan (αSG) expression in αSG null mice after intra-arterial transplantation of wild-type mesoangioblasts. Quadriceps from control (CTR) mice (left image), αSG null (α-SG KO) mice (central image), and treated α-SG null mice (injected with wild-type mesoangioblasts 2 months before sacrifice) (right image). Sections were stained with antibodies against α-sarcoglycan (red), laminin (green) and with 4', 6'-diamidino-2-phenylindole (DAPI) (blue). Inset: Higher magnification of treated muscle with centrally located nuclei. Scale

bar: 100 μm. Adapted from Sampaolesi et al., 2003

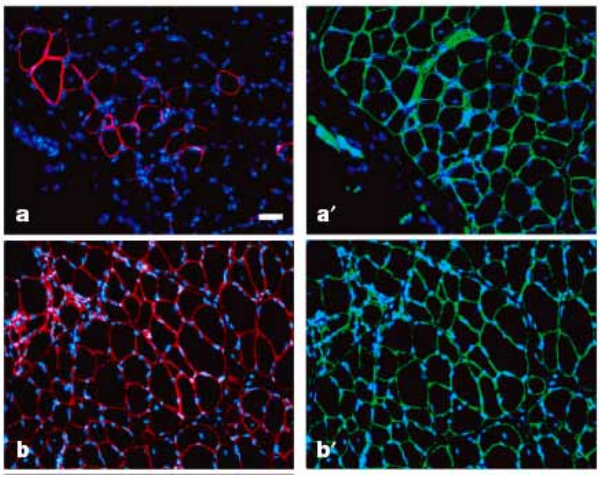


Figure 2.3.2.2 - Dystrophin expression in GRMD dogs treated with MABs transplantation. Immunofluorescence analysis of tissue from treated dogs with autologous genetically corrected MABs (a-a') and with MABs from healthy donor (b-b') revealed fibers (laminin, green) expressing dystrophin (red). Nuclei are stained with DAPI. Scale bar: 100 μm. Adapted Sampaolesi et al., 2006.

Recently, Tedesco and colleagues reported the first evidence of an effective stem cell mediated gene replacement therapy using a human artificial chromosome carrying the entire dystrophin locus (DYS-HAC). The transfer of DYS-HAC into murine dystrophic mdxMABS resulted in genetically corrected cells (mdx(DYS-HAC)MAB), which once injected both intra-muscularly and intra-arterially into *scid/mdx*, are able to ameliorate functionally and morphologically the dystrophic phenotype (Tedesco et al., 2011) .

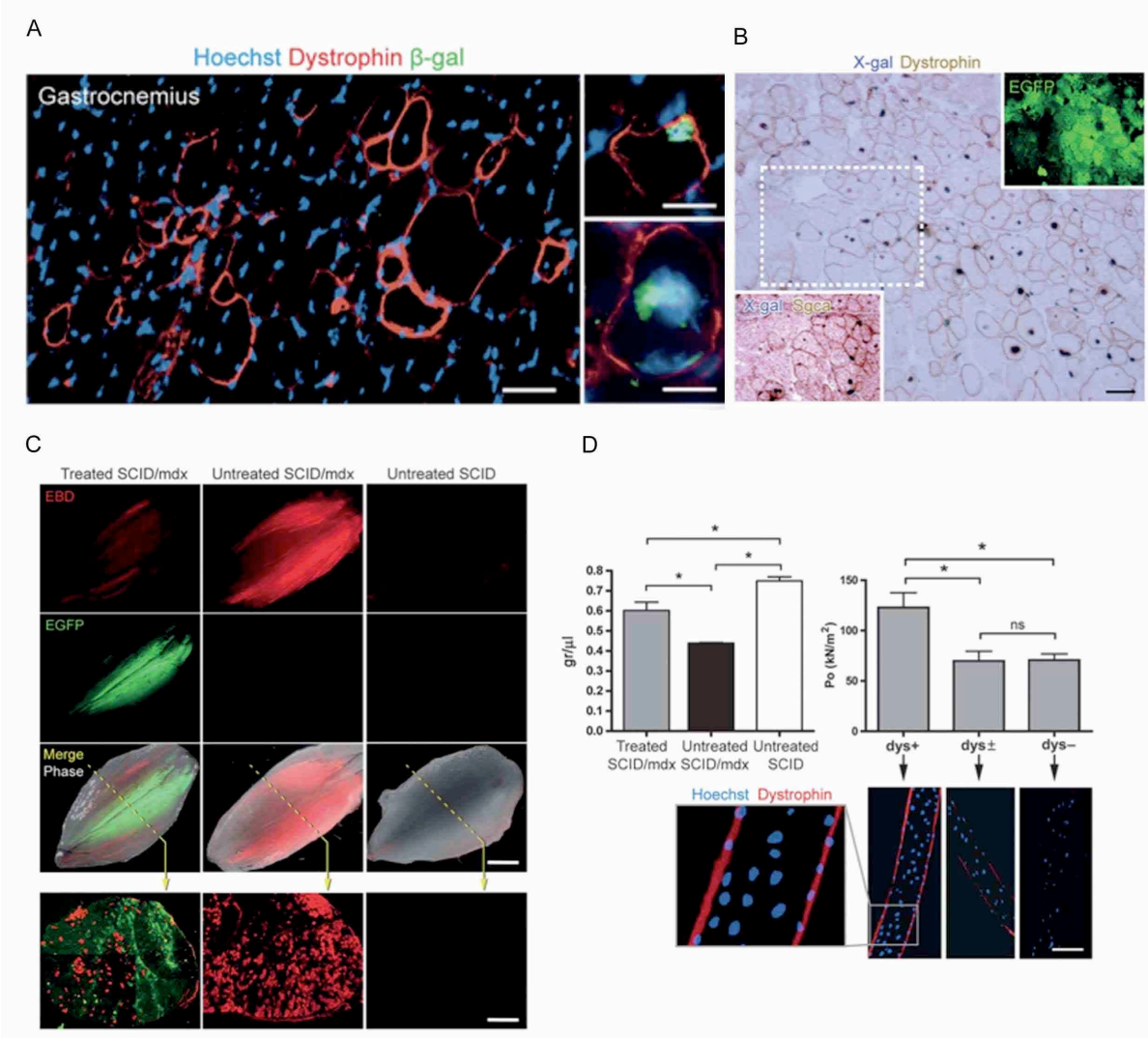


Figure 2.3.2.2 - Morphological and functional amelioration of *mdx* mice after injections with mdx(DYS-HAC) MABs. A) Immunofluorescence images showing dystrophin-positive fibers 3 weeks after intra-arterial transplantation. Right images: dystrophin-positive fibers containing b-gal–positive nuclei; b-gal/dystrophin double-positive fibers were difficult to identify because of cellular dispersion in the entire muscle [scale bars, 50 mm (bottom left), 30 mm (top right), and 20 mm (bottom right)]. Note: mdxMABsDYS-HAC cells are positive for GFP (which is cloned onto DYS-HAC) and X-gal staining (clones used for in vivo experiments were previously infected with a nuclear LacZ lentivirus to increase detection). **B)** Immunohistochemistry showed a cluster of dystrophin-positive fibers, some of which contain X-gal–positive donor nuclei (tibialis anterior muscle), after intra-muscular injections. Upper inset: EGFP fluorescence on the same section. Lower inset: Sgca-positive myofibers in the same section demonstrating the re-assembly of

Dystrophin- glycoprotein complex (DGC) (scale bar, 100 mm). **C)** Evans blue dye (EBD) uptake assay for muscles in mice injected with mdx(DYS-HAC)MABs and after exhaustion on the treadmill test. The upper panel contains fluorescent stereoscopic images of tibialis anterior (TA) muscles. Images show less EBD fluorescence in the treated muscle and no EGFP-EBD co-localization (scale bar, 2 mm). Dashed lines mark the level of the sections indicated by the arrows (scale bar, 1 mm). Transversal sections show less EBD-positive fibers in treated versus untreated muscles. **(E)** Force measurements of transplanted muscles. Left graph: normalized tetanic force of isolated tibialis anterior muscles from intramuscular transplant mice. Right graph: Mean values of specific force for a population of 100 single myofibers dissected from tibialis anterior muscles injected intramuscularly with corrected mdx mesoangioblasts. Myofibers were grouped into dystrophin-positive (dys+; n = 36), dystrophin partly positive (dys±; n = 31), and dystrophin-negative (dys−; n = 33) based on immunostaining after force determination (examples in the bottom row; scale bar, 30 mm). Error bars represent means ± SEM. *P < 0.005; **P < 0.005; ***P < 0.0005, one-way ANOVA. Ns, not significant. Adapted from Tedesco et al., 2011.

Cells similar to murine mesoangioblasts have been isolated from human post-natal skeletal muscle and shown to represent a subset of pericytes and to give rise to dystrophin-positive muscle fibers when transplanted into *scid/mdx* mice (Dellavalle et al., 2007). Based upon these evidences, a phase I/II clinical trial with MAB allo-transplantation in DMD patients is ongoing.

Although the MAB allo-transplantation may represent a real opportunity to cure the MDs, the autologous approach would represent the future and the final aim for the cell-based therapies. Unfortunately, as briefly mentioned above, human MABs undergo senescence thus preventing any in vitro cell manipulation and gene correction.

2.4 TELOMERES, TELOMERASE AND SENESCENCE

2.4.1. Telomeres

2.4.1.1 Telomere structure and function

Since McClintock's and Muller's first descriptions in the 1940s, telomeres have been recognized as important capping structures that play a crucial role in distinguishing the real ends of linear chromosomes from *bona fide* double-stranded DNA (dsDNA) breaks (McClintock, 1941). The DNA sequence of telomeres consists of tandem GT-rich repeats, (Blackburn, 2001), (TTAGGG)n in humans and other vertebrates, with a single-stranded 3-end overhang (Makarov et al., 1997; Wright et al., 1997): electron microscopy analysis has revealed that the single-stranded 3-end overhang penetrate the duplex telomeric DNA repeat array to form a D-loop and T-loop structure *in vitro* (Greider and Blackburn, 1985; Griffith et al., 1999) (Fig.2.4.1.1.1). Telomere binding proteins function to maintain and regulate this typical structure *in vivo*.

In recent years, a large number of studies has shown that telomere function is strictly linked to several essential biological functions such as cell cycle control, cellular immortalization, aging and cancer. Telomeres protect chromosomes from recombination, end-to-end fusion, and recognition as damaged DNA, provide a mean for complete replication of chromosomes and a functional organization of chromosomes in the nucleus; they have an important role in the regulation of gene expression thanks to the so called "telomere positional effect" and serve as a molecular clock that controls the replicative capacity of human cells and their entry into senescence. Nevertheless, there are still a several open questions about the molecular details of how a telomere is

monitored, regulated, and modified and how these functions can permit cell cycle progression (Stewart and Weinberg, 2006).

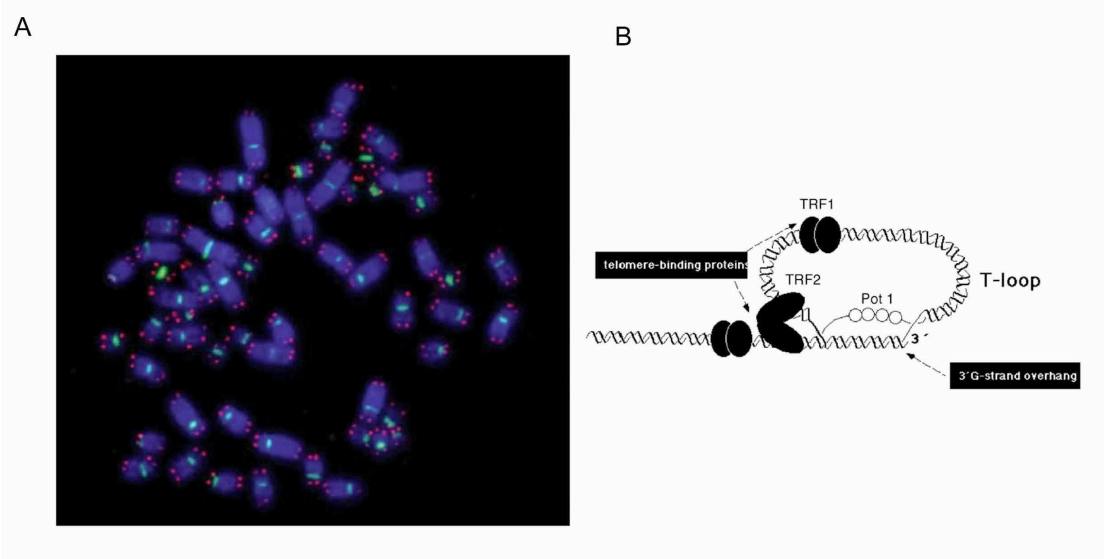


Figure 2.4.1.1 - Telomere structure. A) Telomere (red) and centromere (green) localization of metaphase HeLa chromosomes (Hoechst) by fluorescence in situ hybridization (FISH). **B)** A putative structure of a capped telomere based on the T-loop model. Both telomere-binding proteins and the G-strand overhang are required to maintain telomere capping. Taken respectively from Stewart and Weinberg, 2006 and Blasco, 2003 (Blasco, 2003).

2.4.1.2 Telomere and replicative senescence

In the 1960s, Leonard Hayflick's work about cell proliferative potential created a new field of study. He simply observed that cells could pass through only a precise number of division cycles: cells that had reached this limited cell divisions number, later called Hayflick limit, were termed senescent. Such cells remained metabolically active but were no more able to divide (Hayflick, 1965). Importantly, Hayflick demonstrated that cells isolated from same individuals on multiple occasions, recapitulated this growth phenotype. These suggest that cells have an intrinsic mechanism capable of tracking the number of cell divisions through which they are able to undergo, preventing any further division. This led to the final hypothesis that a limited proliferative capacity plays an important role in aging (Hayflick, 1976).

A link between cell senescence and telomere was suggested in the 1990s, when the so called "telomere hypothesis" was described for the first time (Harley et al., 1990; Allsopp et al., 1992; Levy et al., 1992). Previously, in the 1970s, Watson and Olovnikov independently described the "end replication" problem, in which they suggested that linear chromosomes would be unable to replicate their extreme 3′ ends faithfully (Watson, 1972; Olovnikov, 1973). According to their model, a small portion of chromosomal DNA would be lost following each complete round of replication, creating a problem if codifying sequences were located at the end of the chromosome (Fig. 2.4.1.2.1). This potentially dangerous problem was solved by existence of the telomere (Blackburn, 1991).

To investigate this hypothesis, other researchers examined the telomeric DNA of human chromosomes during successive rounds of DNA replication and cellular division (Allsopp, 1992). They also followed a population of cells

throughout its replicative lifespan and demonstrated that mean telomere lengths were reduced progressively with each subsequent division, exactly as Watson's (1972) and Olovnikov's (1973) models predicted. Most importantly, cells isolated from the same individual and followed in several independent cultures entered into senescence with roughly the same average telomeric lengths. This crucial observation suggested that telomere shortening acts as a genetic clocking mechanism, as described by Hayflick, taking care of divisions number through which an individual cell lineage had passed both *in vitro* and *in vivo*. In addition, it was hypothesized that once the telomeres shortened to a certain predetermined length, these DNA sequences were responsible for triggering entrance into senescence. All these results changed the scenario of chromosome replication setting the basis, together with the discovery of telomerase, for our current understanding of the link between telomere erosion, senescence and human aging. These brilliant results earned to Carol Greider, Elizabeth Blackburn and Jack Szostack the Medicine Nobel prize in 2009.

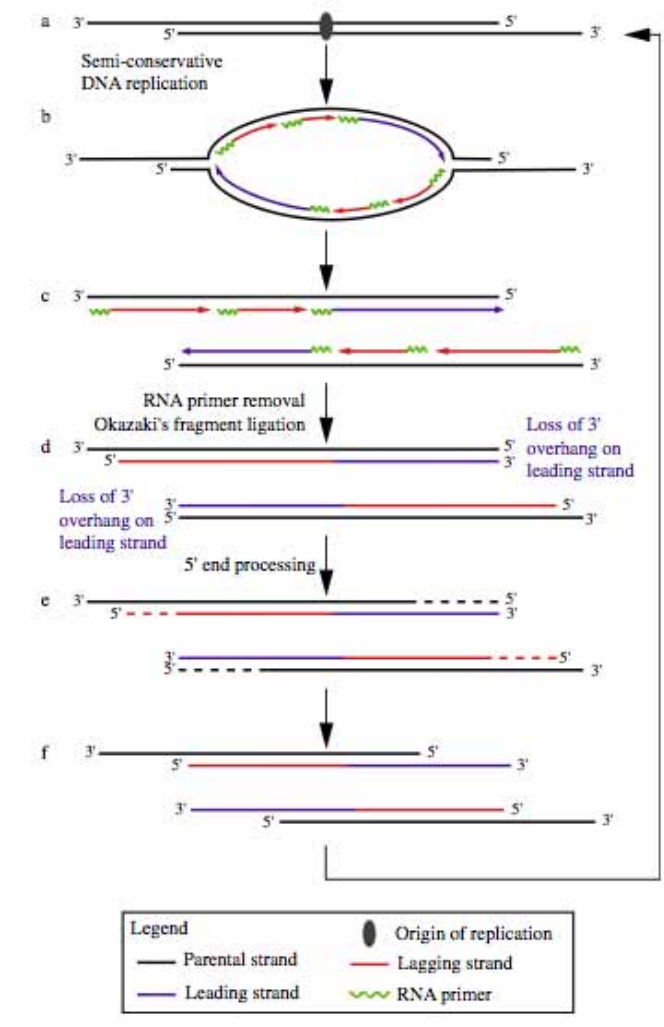


Figure 2.4.1.2.1 - The DNA end replication problem. DNA replication starts at replication origins (a, black oval). The replication forks move in opposite directions (b). Because DNA polymerases only elongate in the 5′ to 3′ direction, each fork contains a leading (continuous) and a lagging (discontinuous) strand. Lagging strand synthesis is primed by short RNA oligonucleotides (wavy line). Terminal DNA is lost at the leading strand end because the parental 5′-end-containing template is recessed (d). If RNA primers were laid down on the 3′ overhang as depicted in (c), their removal would not lead to a net loss of sequence at the lagging strand. Finally, 5′-end processing occurs to regenerate a 3′ overhang at the leading strand telomere (e, f). Taken from Hug and Lingner, 2006 (Hug and Lingner, 2006).

2.4.1.3 Cell senescence and human aging

Currently, there are two non-mutually exclusive hypotheses to explain the molecular basis of cellular and, eventually, of organism aging (Aviv, 2004; Ben-Porath and Weinberg, 2004; Vijg and Suh, 2005). The first suggests that aging is the result of the slow accumulation of damage that leads to cellular and eventually tissue deterioration. The other suggests that aging is the consequence of an intrinsic program, which is finely regulated by a biological clock, such as telomere length (Figure 2.4.1.3.1). As described above, *in vitro* human cells undergo a defined number of cellular divisions before undergoing replicative senescence, a state in which cells are still viable but not able to divide anymore.

The role of telomere homeostasis and cell senescence in human aging has been supported by studies demonstrating a relationship between donor age and telomere lengths, correlations between *in vitro* growth capacity and donor age, and reduced *in vitro* growth capacity of cells isolated from patients suffering from various types of premature aging (progeria) when compared with normal, age-matched control cells

(Martin et al., 1970; Lindsey et al., 1991; Faragher et al., 1993; Dimri et al., 1995). Although interesting and convincing and largely accepted, the work on telomere by Carol Greider, Jack Szostack and Elizabeth Blackburn has not yet conclusively demonstrated that telomere erosion is the primary causal force that drives human aging, especially for those tissue, such as muscle or brain, where differentiated cells do not divide.

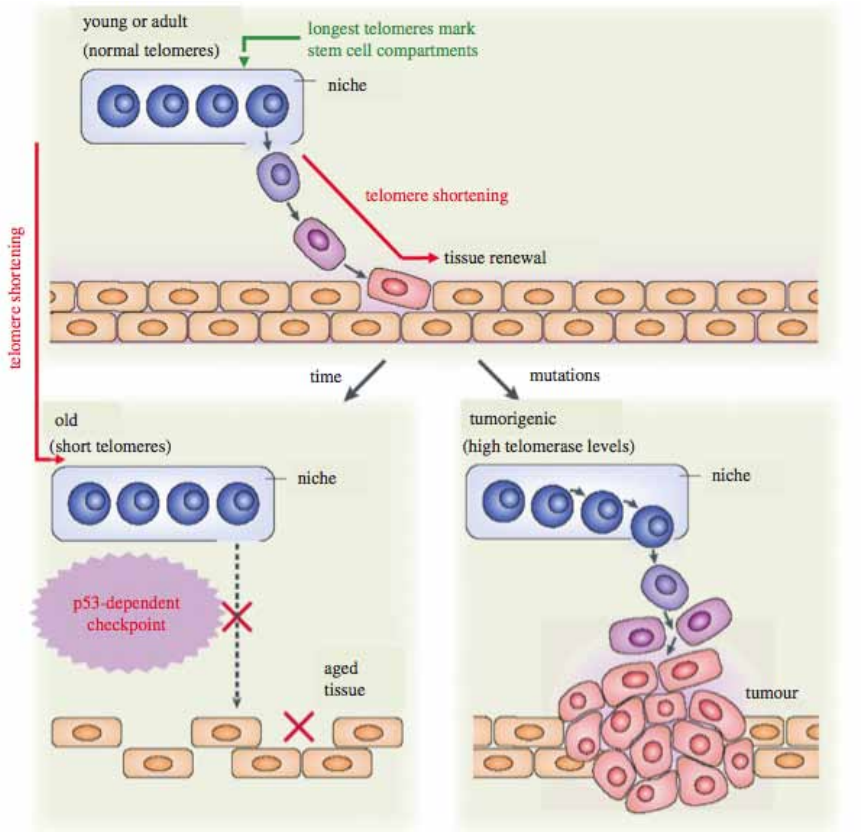


Figure 2.4.1.3.1 - Model for the role of telomeres in cancer and aging. Long and constant telomeres are a typical feature of stem cells. In young or adult organisms, stem cells (blue rounded cells) repopulate tissues (square orange cells). During this process, stem cells undergo telomere shortening, which is partially counterbalanced by the action of telomerase. In old organisms, stem cell telomeres critically shorten being recognized as DNA damage. If the stem cells express aberrantly high levels of telomerase, stem cell mobilization is more efficient than normal. Under these higher mobilization conditions, tissue fitness would be maintained for a longer time, increasing lifespan and also the probabilities of initiating a tumour. Taken from Donate and Blasco, 2011 (Donate and Blasco).

2.4.2 Telomerase

2.4.2.1 Telomerase structure and function

Telomerase is a RNA-dependent DNA polymerase enzyme that is able to synthesize and to add the hexameric repeats, TTAGGG, to chromosome ends, maintaining the length of the telomeres and thereby extending the number of cell divisions providing in this way the molecular basis for unlimited proliferative potential (Blackburn, 1992; Holt and Shay, 1999; Shay and Wright, 2001).

Telomerase consists of two essential components: one is the functional RNA component (in humans called hTR) (Feng et al., 1995), a template for telomeric DNA synthesis; the other is the catalytic protein (hTERT) that has a reverse transcriptase activity (Harrington et al., 1997b; Kilian et al., 1997; Lingner et al., 1997; Meyerson et al., 1997) (Fig. 2.4.2.1.1). hTR is highly expressed in all tissues regardless of telomerase activity (Shay and Wright, 1999) whereas the expression of the human catalytic component hTERT is closely associated with telomerase activity (Yi et al., 1999). hTERT is generally repressed in normal cells and upregulated in immortal cells, suggesting its primary role in determining the enzymatic activity. The telomerase gene was recently mapped to 5p15.33 as one of the most distal genes on chromosome 5p (Shay and Wright, 2000).

Since its first discovery in 1985 in Tetrahymena thermophila (Greider and Blackburn, 1985), telomerase activity was found to be absent in most normal human somatic cells but present in over 90% of cancerous cells and *in vitro* immortalized cells (Kim et al., 1994; Shay and Bacchetti, 1997). A big number of studies showed that expression of human telomerase alone is sufficient for the

immortalization of different cellular types (Bodnar et al., 1998; Ramirez et al., 2001). Indeed, the introduction of the catalytic protein (hTERT) component of telomerase into normal fibroblasts and epithelial cells prevents shortening of the telomeres resulting in immortalization (Ramirez et al., 2001). The key role of telomerase in inducing immortalization is the maintenance of telomere length, not to produce a net increase in length (Shay and Wright, 2001). Transient expression of a *cre*-excisable telomerase results in a preferential lengthening of the shortest telomeres and an increase in lifespan proportional to the length of the shortest telomere (Steinert et al., 2000). Likewise, the inhibition of telomerase in immortalized human cells leads to progressive telomere shortening and cell death (Herbert et al., 1999). These results demonstrate that telomerase plays an important role in cellular aging and cancer, underlying the driving role of telomerase activity in the developing of diagnostic and therapeutic strategies.

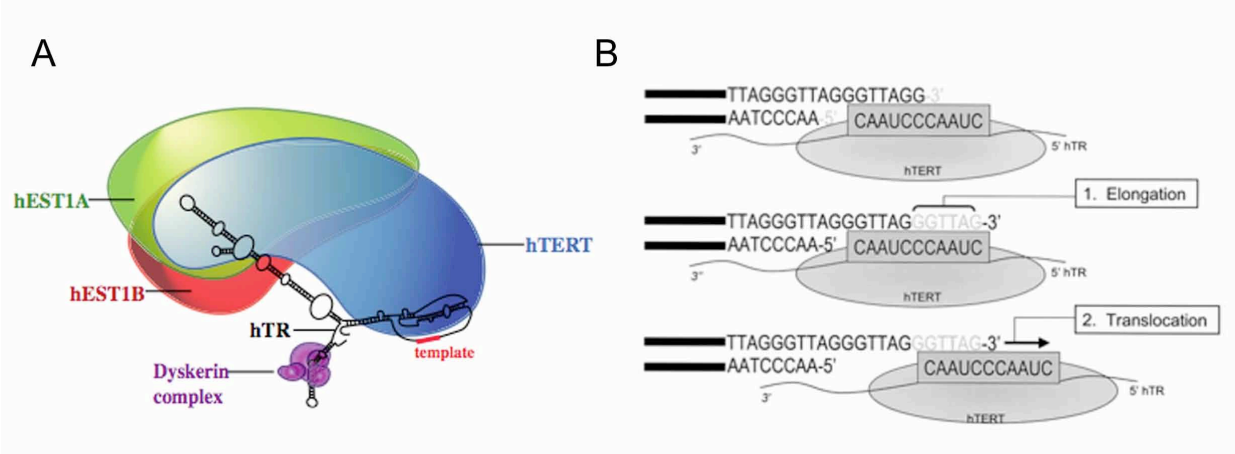


Figure 2.4.2.1.1 - Telomerase holoenzyme structure and maintenance of telomere length. **A)** Homo sapiens telomerase model: telomerase RNA secondary structure is based on the published model (Chen et al. 2000). A large set of additional proteins, which are not represented,

has been reported to interact with human telomerase. Taken from Hug and Lingner , 2006. **B)** The telomerase holoenzyme adds telomeric repeats, TTAGGG, in two successive steps: elongation (1) and translocation (2). The enzyme is composed of two main components: hTR is the telomerase functional or template RNA portion, and hTERT is the telomerase reverse transcriptase enzymatic portion. The telomeric end can binds to the template region of hTR and is elongated by the addition of the bases complementary to the template via the catalytic subunit (hTERT). The complex then pauses and translocates and repeats the elongation of the telomere (e.g. the human telomerase complex is processive). Taken from Granger et al., 2002 (Granger et al., 2002).

2.4.2.2 Telomerase, senescence and immortalization

As described above, every time the chromosome is replicated during each cell division, the result is the progressive loss of DNA at the chromosomal ends (Lodish et al., 1995). Cells that have reached their division limit (Hayflick limit), as determined by telomere length, undergo a process called 'replicative senescence' or 'mortality stage 1' (M1), in which cells are still alive and metabolically active but are no longer able to divide (Harley et al., 1990; Dimri et al., 1995). It may happen that cells succeed in bypassing senescence through inactivation of the p53 and/or Rb pathways, thus compromising genetic stability since telomeres continue to shorten becoming unable to protect the real chromosome ends. When this occurs, cells subsequently enter a second proliferative block referred to as crisis or mortality stage 2 (M2), which is characterized by short telomeres, end-to-end chromosomal fusions, anaphase bridges, and cell death by apoptosis (Counter et al., 1992; Shay and Wright, 2005). On occasion, however, a rare clone can survive from crisis; such cell clones are considered immortal (Fig. 2.4.2.2.1). Analysis of telomeric DNA in these clones indicate that telomere lengths are maintained despite the high

number of cell divisions (Wright and Shay, 1992).

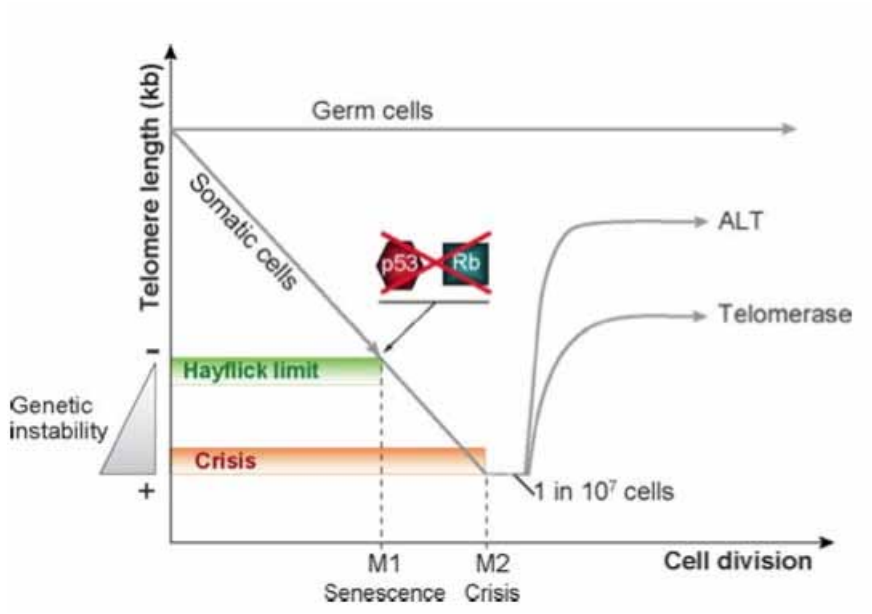


Figure 2.4.2.2.1 - The telomere hypothesis. Telomere length (ordinate) is progressively lost during successive rounds of cellular division (abscissa), eventually leading to p53- and Rb-dependent permanent growth arrest, referred to as senescence. Inactivation of p53 and Rb function allows continued cellular division and further telomere shortening. Telomeres eventually erode to a length at which they are unable to protect chromosome ends, resulting in crisis, i.e., end-to-end chromosome fusions and apoptotic cell death. Rare clones (1 in 107) may emerge from a population of cells in crisis. These clones maintain stable telomere lengths through the activation of a telomere maintenance mechanism, i.e., human telomerase catalytic subunit (hTERT) expression or the alternative lengthening of telomeres (ALT) mechanism. Taken from Stewart and Weinberg, 2006.

However, escape from senescence and acquisition of an indefinite life span is an exceptionally rare event in human cells (1×10^{-7}), so they are limited in their lifespan (Campisi et al., 2001). This limitation has hampered progress in isolation and expansion of tissue-specific progenitor or stem cells from different

tissues, indeed putting back also the possibility to largely employ it for experimental and/or therapeutic purposes.

For these reasons, in the last years many groups tried to immortalize different human cell types *in vitro* by forcing the expression of different genes. Cell lines so obtained can be expanded, cloned and extensively characterized.

Initially it was shown that the exogenous expression of viral oncoproteins, such as SV40 large T antigen (T-Ag), which acts through binding of Rb and p53 (Ali and DeCaprio, 2001) can only extend the replicative lifespan of human cells, but does not lead to actual immortalization. In the case of human myoblasts, expression of TAg produces chromosomal rearrangements reduces and delays cell fusions interfering at the end with myogenic differentiation (Mouly et al., 1996). It was discovered that the introduction of the cDNA coding for hTERT into human fibroblasts, retinal pigment cells and endothelial cells was sufficient to trigger telomerase activity, to block telomere shortening and to maintain cells in proliferation beyond their usual lifespan (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Chaouch et al., 2009). Tumour derived cell lines with telomerase activity usually showed limited and usually abnormal differentiation. However, works performed both on bone marrow stromal and endothelial cells showed that hTERT expression provokes an increase in their life span without hampering their differentiation potential (Yang et al., 2001; Shi et al., 2002)

On the other hand, telomerase alone was not sufficient to immortalize different cell types: classically, genes encoding for at least two types of proteins have been used to immortalize human cells. First, is necessary to overcome the telomere shortening-related senescence with the introduction of hTERT and so reconstituting telomerase activity. Second, the use of proteins, such as Bmi-1

and cyclin-dependent kinase 4 (cdk4), that promote cell cycle progression (e.g., growth promoters or cell cycle inducers). In both cases, these proteins avoid senescence due to p16-mediated stress response (Ramirez et al., 2001): Bmi-1 downregulates the p16 and p19Arf tumour suppressor genes encoded by the *ink4a* locus (Jacobs et al., 1999), whereas the over-expression of cdk4 counteract the p16 effect. So, it has been demonstrated that is possible to successfully immortalize human cells by the expression of two genes, hTERT and a cell-cycle promoter such as cdk4 or Bmi-1 (Cudre-Mauroux et al., 2003; Ramirez et al., 2004; Saito et al., 2005; Zhang et al., 2006; Zhu et al., 2007).

3. RATIONALE

DMD is a neuromuscular genetic disease which primarily affects skeletal muscles leading to progressive paralysis and premature death (Emery, 2002; Emery and Muntoni, 2003). Although mutations responsible of DMD were identified several decades ago in the dystrophin X-linked gene (Kunkel et al., 1985; Ray et al., 1985) and new gene therapy and stem cell transplantation strategies are under investigation (Tedesco et al., 2010; Goyenvalle et al., 2011), so far there are no successful and definitive treatments.

Some years ago, a population of vessel–associated progenitors called mesoangioblasts (MABs) has been isolated and characterized showing that these cells are able to differentiate into skeletal muscle and to cross the vessel wall and the basal lamina of skeletal myofibers, offering the possibility to be injected into the arterial circulation for cell therapy protocols. As a consequence, the intra-arterial administration of both donor and genetically corrected MABs into different pre-clinical models of muscular dystrophy, resulted in a functional and morphological amelioration of dystrophic phenotype (Koenig et al., 1987; Galvez et al., 2006; Sampaolesi et al., 2006; Gargioli et al., 2008; Diaz-Manera et al., 2010; Tedesco et al., 2011). Cells similar to mesoangioblasts have been isolated from human post-natal skeletal muscles (human MABs), characterized as a subset of pericytes with myogenic potency (Dellavalle et al., 2007). These features, together with the observations that these cells are expandable in culture and can be easily transduced with therapeutic viral vectors, lead human MABs to be considered an eligible population for the gene and cell therapy of muscular dystrophies. Moreover, this extensive pre-clinical work set the

conditions for a Phase I/II clinical trial based upon human MABs intra-arterial allogeneic transplantation (EudraCT no. 2011-000176-33), currently ongoing at San Raffaele Hospital in Milan.

However, transplantation of autologous genetically corrected muscle progenitor cells, would be preferable because immune suppression would not be required. On the other hand, there are still limitations for autologous cell therapy approach. One of the major challenge is represented by the fact that the dystrophin gene is the largest of human genome (genomic locus 2.4Mb, cDNA 14Kb) (Koenig et al., 1987; Muntoni et al., 2003), preventing its accommodation into viral vectors for efficient gene delivery. In this direction, alternative gene-replacement technologies have been developed and tested (Arnett et al., 2009) (see INTRODUCTION for details). All these strategies are promising but present disadvantages; exon-skipping and nonsense codon suppression cannot be used for all DMD mutations, just to mention a few.

Human artificial chromosomes (HACs) are optimal candidate to overcome these obstacles (Kazuki and Oshimura, 2011). They can contain large genomic regions with regulatory elements and remains episomal avoiding the risk of insertional mutagenesis. For this reason, a human artificial chromosome containing the entire dystrophin locus (DYS-HAC) has been developed, giving rise to the possibility to employ it for DMD cell-/gene-therapy (Hoshiya et al., 2009). Recently, the transfer of the DYS-HAC into murine dystrophic mesoangioblasts (mdxMABs) generated genetically corrected cells (mdx(DYS-HAC)MABs) which significantly ameliorated muscular dystrophy upon transplantation into *mdx* mouse (Tedesco et al., 2011).

These results clearly showed the feasibility and the potential of this strategy

providing the basis for translating it into human cells. Nevertheless, human MABs will require an additional step to extend their proliferative capability, since they undergo replicative senescence upon long-term culture. As a matter of fact, an unlimited proliferative potential would ensure their survival after HAC transfer and selection, as already seen in human mesenchymal stem cells (Hoshiya et al., 2009). In this direction, the attention was focused to the development of a platform for engineering human MABs from both healthy donor and DMD patients containing excisable lentiviral vectors expressing immortalizing genes in a reversible fashion, such as hTERT, the catalityc subunit of telomerase, and Bmi-1, a cell cycle promoter (Salmon et al., 2000; Cudre-Mauroux et al., 2003). After verifying the feasibility and stability of the immortalization on procedure *in vitro* and *in vivo* using human MABs from healthy donors, DMD MABs have been successfully immortalized and genetically corrected with the DYS-HAC.

This work demonstrate translation of HAC-based gene-correction strategy to a human clinically relevant stem/progenitor cell population, reinforcing hopes for future DMD autologous therapy.

4. RESULTS

4.1 hTERT AND Bmi-1-MEDIATED IMMORTALIZATION OF HEALTHY DONORS HUMAN MABs

4.1.1 Isolation and lentivector-mediated immortalization of healthy donor-derived MABs

To investigate the possibility to achieve an unlimited proliferative potential through the combined expression of hTERT and Bmi-1, human MABs have been isolated from muscle biopsies of three different healthy donors: a 72 years old female (healthy donor #1), a 22 years old female (healthy donor #2) and a 53 years old male (healthy donor #3). The different populations of cells obtained from muscle explants were FACS-sorted to separate MABs (AP+/CD56-) from myoblasts (AP-/CD56+) and fibroblasts (AP-/CD56-) (Fig. 4.1.1.1A).

AP+/CD56- sorted cells showed the typical MAB morphology appearing as small, triangular and adherent to the substrate with a number of floating cells which represented the proliferating fraction (Fig. 4.1.1.1B). Each population (healthy donor#1, #2 and #3) was then co-transduced with floxable hTERT and Bmi-1 lentiviral vectors at passage 3. In parallel MABs from each donor were transduced with a floxed GFP lentiviral vector to obtain a control population. hTERT/Bmi-1 and GFP polyclonal populations derived from healthy donor#1 were then cloned by limiting dilution.

Three hTERT+Bmi-1 clones were selected as well as GFP clones, one of this, GFP#B5, was used as a control (Fig. 4.1.1.1C-G). Selection of

hTERT/Bmi-1 expressing clones was performed evaluating both telomerase activity (due to hTERT catalytic subunit) and Bmi-1 expression. Indeed it is known that human MABs after more than 10 passages in culture showed down-regulation of telomerase activity (Dellavalle et al., 2007). As expected, Telomeric Repeat Amplification Protocol (TRAP) revealed that hTERT/Bmi-1 MABs had high levels of telomerase activity whereas GFP#B5 did not (Fig. 4.1.1.1C). Bmi-1 protein expression levels were verified by Western Blot. As showed in Fig. 4.1.1.1D, Bmi-1 expression was clearly present only in hTERT/Bmi-1 clones; conversely, Bmi-1 protein was not detected in GFP control clones after the same number of passages in culture.

Moreover, morphological analysis of hTERT/Bmi-1 #A1, #B2 and #C5 clones suggested that exogenous expression of the catalytic subunit of telomerase hTERT and of the cell cycle promoter gene Bmi-1, did not alter the morphology of human MAB clones (Fig. 4.1.1.1E), which resembled the one of the paternal population (Fig. 4.1.1.1B, top left image).

Proliferation ability of clones was assayed in terms of population doublings (PD) and BrdU incorporation. PD curves demonstrated that the combined expression of hTERT and Bmi-1 allowed human MABs to bypass the early senescence that normally occurs *in vitro*. Conversely, the GFP clone underwent senescence, as already observed for human primary MABs (Dellavalle et al., 2007) (Fig. 4.1.1.1F). These results were confirmed by BrdU incorporation assay; 1 hour pulse was performed at different passages/time points, showing a high proliferation rate for all the hTERT/Bmi-1 expressing clones but not for the GFP one (Fig. 4.1.1.1G). Moreover the rate of proliferation seem to be maintained stable with time, albeit at the first time point (p12) two of

three hTERT/Bmi-1 expressing clones (#A1 in blue and #C5 in orange) showed a lower rate of proliferation which is again increased in the subsequent time points (respectively p40 and p70) (Fig. 4.1.1.1G). This result was probably due to the fact that these clones bypassed a phase of proliferative crisis which had occurred at that passage and from which they exited re-starting to proliferate robustly later in time.

In conclusion the combined expression of hTERT and Bmi-1 leads to a functional immortalization of human MABs, which retain their normal behaviour and continue to proliferate in culture with a stable proliferation rate.

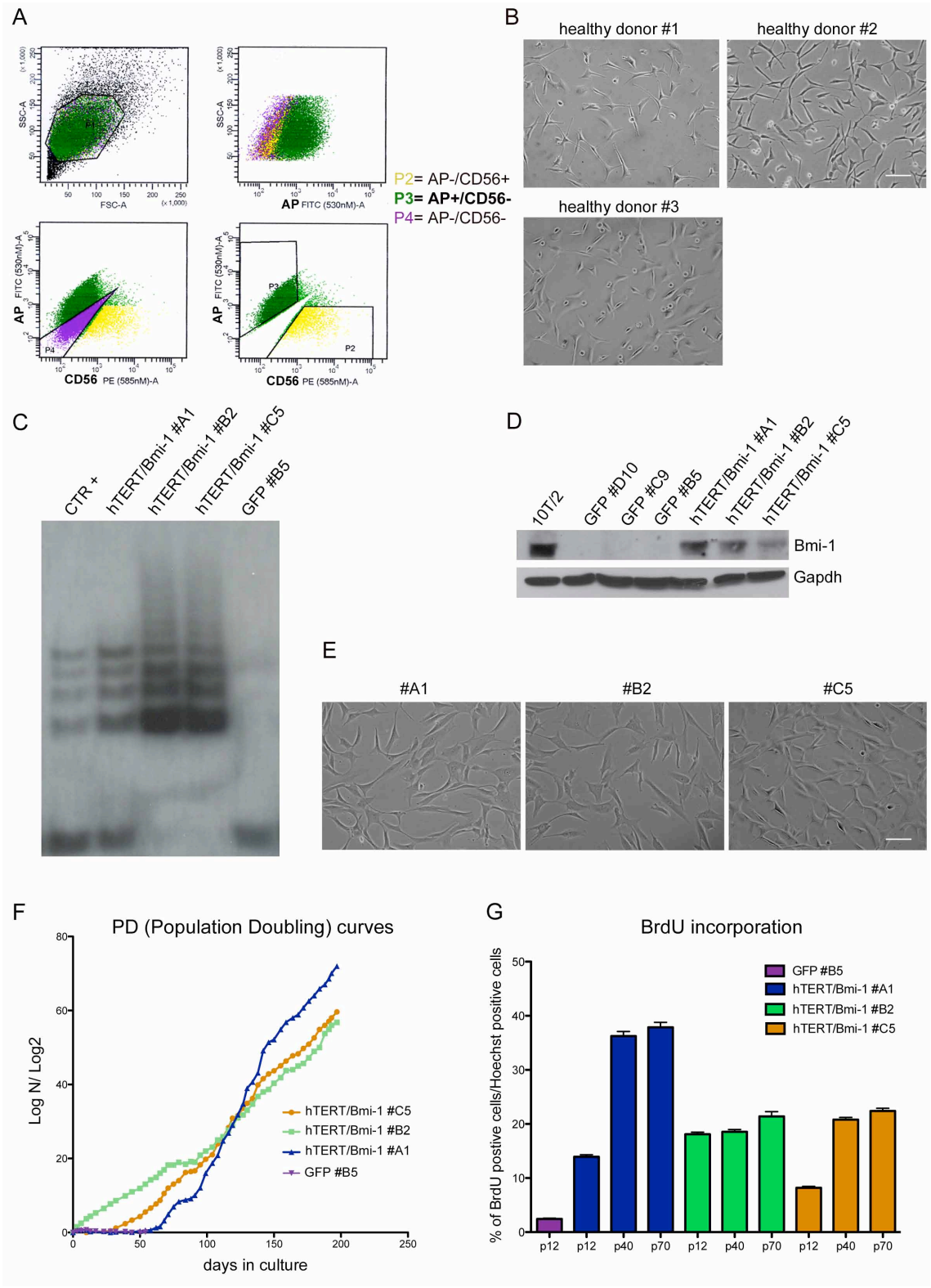


Figure 4.1.1.1 - hTERT and Bmi-1 mediated immortalization of human MABs.

A) Representative example of FACS-sorting analysis of a mixed population (myoblasts, fibroblasts and MABs) obtained from muscle explants. Cells were analyzed for their physical

parameters and then for the levels of conjugated fluorocromes AP-FITC and CD56-PE: only AP+/CD56- cells (P3) were sorted in order to obtain MABs without fibroblasts (P4) or myoblasts-derived cells (P2) contamination. B) Phase contrast morphology of sorted MABs derived from three different healthy donors after 3 passages in culture (72 years old female = healthy donor #1; 22 years old female = healthy donor #2; 53 years old male = healthy donor #3). C) Telomeric Repeat Amplification Protocol (TRAP) performed on hTERT/Bmi-1 clones showing high telomerase activity which is not present in the control clone (GFP #B5). The figure shows gel containing a ladder of PCR products with 6 base increments per sample, each one corresponding to a telomeric repeat added by telomerase (i.e., the number of bands correlates with the telomerase activity). HeLa cervical carcinoma cells were used as positive control. D) Western Blot showing Bmi-1 expression (triplet 40-44 kDa). hTERT/Bmi-1 expressing clones presented Bmi-1 expression, not detected in control GFP clones. As positive control murine fibroblasts (10T/2) were used, whereas GAPDH was used as normalizer (37 kDa). E) Phase contrast morphology of hTERT/Bmi-1 #A1, #B2 and #C5 clones. F) Proliferation curves (Population Doubling = PD) of hTERT/Bmi-1 and GFP clones demonstrating that human MABs co-transduced with hTERT and Bmi-1 bypassed early replicative senescence phase. G) Bar graph depicting proliferation of hTERT/Bmi-1 expressing MABs compared to GFP MABs as BrdU incorporation rate. Scale bar: 50 μm

4.1.2 Analysis of telomeric lengths of immortalized healthy donor MABs

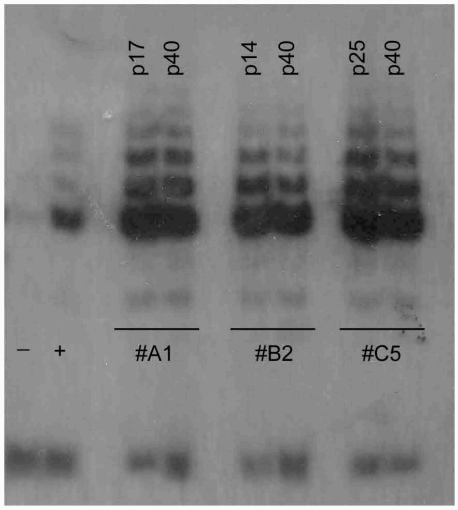
As previously showed (see section 4.1.1, Fig. 4.1.1.1C), the introduction of hTERT into human MABs restored telomerase activity. As a matter of fact, it is known that in humans telomerase activity is turned off during lat embryogenesis and foetal development, being absent in the majority of adult somatic cells (see INTRODUCTION, section *2.4.2.1*). In all cases, expression of the catalytic subunit of telomerase hTERT is turned off, whereas the RNA component hTR is expressed regardless of telomerase activity and, as a consequence, only hTERT expression is directly associated with telomerase activity (Shay and Wright, 1999); for this reason only hTERT was forced in restored in human MABs to promote telomerase reconstitution.

Indeed, after hTERT introduction a TRAP assay was performed on hTERT/Bmi-1 expressing clones at early and late passages to test the actual stability of hTERT transgene expression (under CMV promoter). The results showed that hTERT expression allows to a fully functional and stable reconstitution of telomerase (maintained also after 40 passages *in vitro*; Fig. 4.1.2.1A).

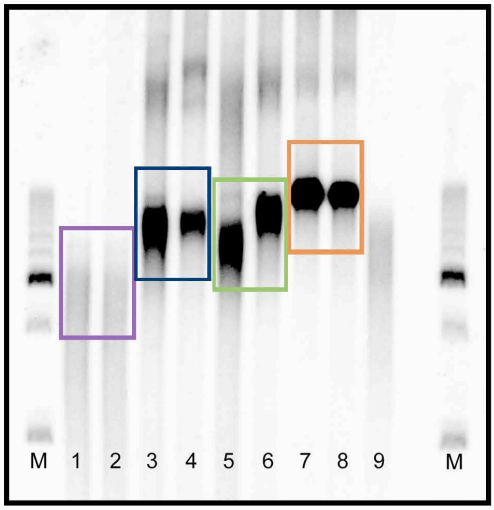
Moreover, to validate the specificity of telomerase activity, its direct target were analyzed: the telomeres. To investigate this, I performed a Telomeric Restriction Fragments (TRF) assay, which is a Southern blot based upon a probe able to specifically recognize telomeric sequences. As expected, hTERT/Bmi-1 clones showed longer telomeres compared with control GFP clone, demonstrating that telomerase is active on its target (Fig. 4.1.2.1B). In addition, two out of three clones, hTERT/Bmi-1 #A1 and #C5, telomeric length

was stably maintained in time, although in the case of hTERT/Bmi-1 #C5 telomeres appeared to be slightly longer.

A



B



C

Number	Sample	Telomeric length (Kb)
1	GFP #B5 p5	6.73
2	GFP #B5 p15	6.54
3	#A1 p17	15.86
4	#A1 p40	17.51
5	#B2 p14	12.78
6	#B2 p40	18.83
7	#C5 p25	29.58
8	#C5 p40	27.81
9	CHQ std	10.5

Figure 4.1.2.1 - Analysis of telomerase activity stability and of telomeric lengths. A) TRAP (Telomeric Repeat Amplification Protocol) assay of hTERT/Bmi-1 #A1, #B2 and #C5 clones at early (respectively at p17, p14 and p25) and late (at p40) passages showing a stable telomerase activity. HeLa cells were used as positive control and CHAPS buffer as negative one. B) TRF (Telomeric Restriction Fragments) southern blot was performed on hTERT/Bmi-1 clones to verify the effect of telomerase on telomeric lengths. M) 2.5 Kb ladder; 1) GFP #B5 p5; 2) GFP #B5 p15; 3) hTERT/Bmi-1 #A1 p17; 4) hTERT/Bmi-1 #A1 p40; 5) hTERT/Bmi-1 #B2 p14; 6) hTERT/Bmi-1 #B2 p40; 7) hTERT/Bmi-1 #C5 p25; 8) hTERT/Bmi-1 #C5 p40; 9) CHQ standard. C) Quantification of telomeric lengths.

4.1.3 *In vitro* and *in vivo* safety of hTERT/Bmi-1 immortalized MABs

Since a strong telomerase activity and a high proliferation rate are typical features of transformed cells, it was opportune to thoroughly investigate both *in vitro* and *in vivo* if the introduction of hTERT and Bmi-1 into human MABs led them to a cancerous transformation.

Two classical *in vitro* assays were performed to test the growth inhibition by cell-cell contact (Fig. 4.1.3.1A) and the growth factor dependence (Fig. 4.1.3.1B) of hTERT/Bmi-1 expressing MABs. In both cases, proliferation was tested in terms of BrdU incorporation (1 hour pulse) 4, 8 and 12 days after the beginning of the experiments. In cell contact inhibition assay, there was a significant decrease in the proliferation, starting from 4 days in culture; in particular, the decrease was stronger after 12 days, when cells were almost confluent (Fig.4.1.3.1A). Similar results have been obtained with the growth factor dependence assay; in this case the reduction of BrdU incorporation rate was even more striking and rapid (Fig.4.1.3.1B).

These parameters were instead not maintained by cancer cells, such as HeLa cells (Fig. 4.1.3.1C). These results demonstrate that the immortalization process does not lead to *in vitro* transformation of human MABs, which retain their normal features, among these the capacity to be inhibited in the growth by cell contact and by the absence of growth factors and serum in the culture medium.

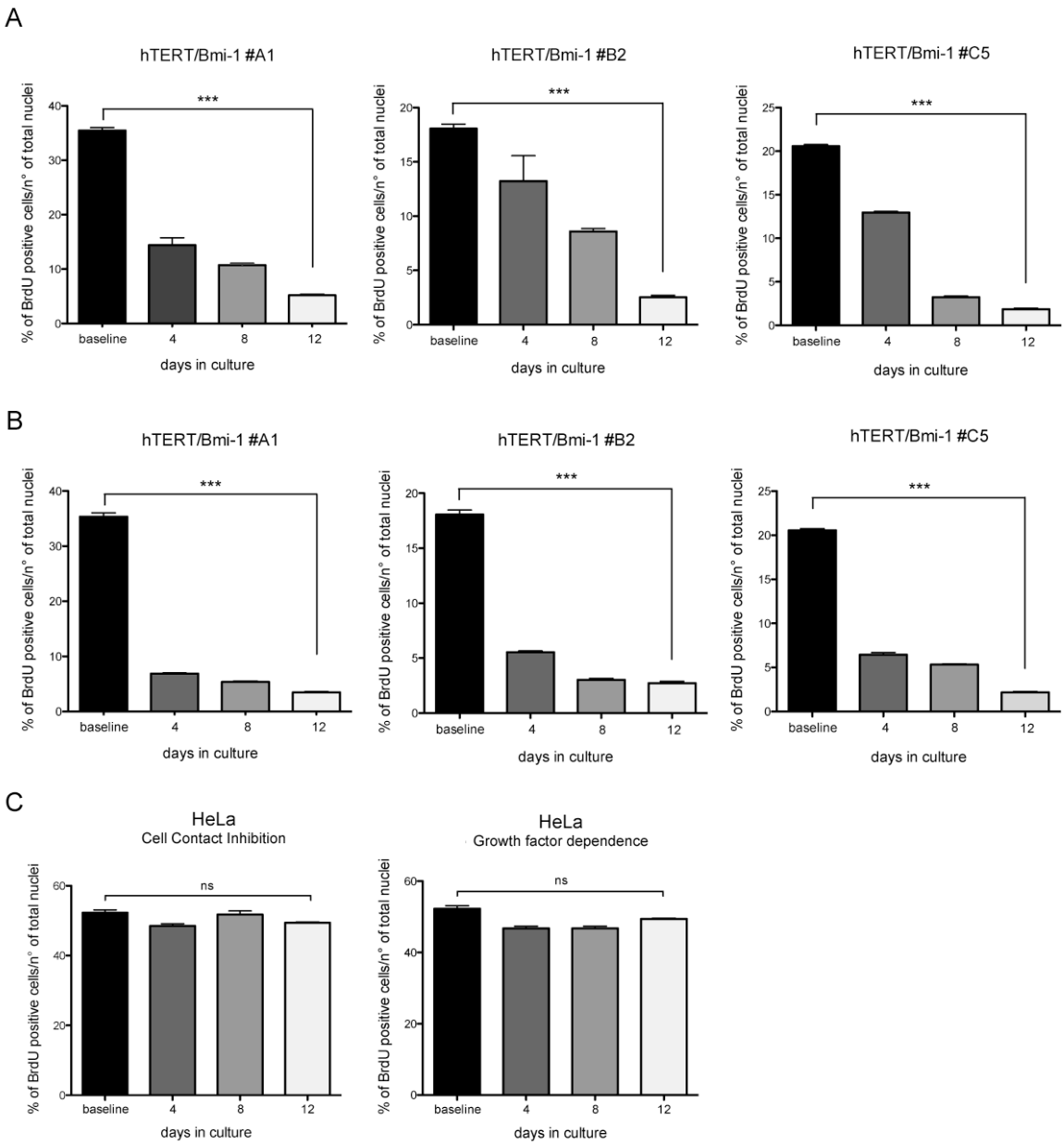


Figure. 4.1.3.1 - *In* vitro safety of hTERT/Bmi-1 immortalized MABs. A, B) Graph bar representations of BrdU incorporation showing responsiveness to the growth inhibition by cell contact (A) and growth factor dependence (B) of human hTERT/Bmi-1 expressing MAB clones compared to HeLa cells (C). Baseline = day after plating cells. ***, $P \leq 0.0005$; ns, statistically not significant.

Finally, the *in vivo* tumorigenicity of hTERT/Bmi-1 immortalized MABs was tested. In order to investigate it, single subcutaneous injections of 2 x 10^6 cells were performed into immunodeficient *scid* mice and 4 mice were used for each hTERT/Bmi-1 #A1, #B2, #C5 clone and for hTERT/Bmi-1 polyclonal populations derived from healthy donor#2 and #3. In parallel, *scid* mice were injected with HeLa cells as positive control. The assay showed that immunodeficient mice injected with hTERT/Bmi-1 immortalized MABs did not develop any tumour after 12 months of follow up, whereas mice injected with HeLa exhibited prominent tumours in less than 3 weeks (data not shown). Results obtained both *in vitro* and *in vivo* demonstrate absence of tumorigenic potential of immortalized human MABs.

4.1.4 Cre-mediated excision of the floxed transgene

To test the functionality of the floxed lentiviral vectors in transgene excision, human MABs from healthy donor #3 were transduced with HLox.CMV.GFP.IRES.TK lentiviral vector and a 100% GFP positive polyclonal population was obtained (Fig. 4.1.4.1C). The floxed GFP vector was chosen for this assay since it was used as backbone to construct the immortalizing floxed lentiviruses hTERT and Bmi-1 we employed for this project, HLox.CMV.hTERT.IRES.TK and HLox.CMV.Bmi-1. Both lentiviral vectors have been constructed to guarantee the reversibility through two safety levels: Cre-LoxP and TK-ganciclovir systems. First, transgenes are flanked by LoxP sites allowing their removal after Cre-recombinase expression. In a second moment, the fraction of cells that have escaped the Cre-excision of the transgene will be killed by the addition in the culture medium of ganciclovir. Ganciclovir molecule

becomes active only after its phosphorylation by viral timidine kinase (TK) and the active form inhibits viral DNA polymerase; as a consequence the administration of ganciclovir will kill all the cells that express the TK suicide gene. In our case, TK gene is cloned between LoxP sites and in IRES with the transgene of interest (hTERT, Bmi-1 and GFP) (Fig. 4.1.4.1A). This will allow to kill cells which did not undergo Cre-mediated transgene excision thus reverting the immortalizing status (Salmon et al., 2000; Cudrè-Mauroux et al., 2003).

To verify the efficiency of the Cre-LoxP system, two different concentration of Cre-recombinase non-integrating lentiviral vector (Fig. 4.1.4.1B) were used on healthy donor#3 GFP MAB population (100% of GFP cells) (Fig. 4.1.4.1C). Three weeks after infection, cells were analyzed by FACS to quantify the number of GFP positive cells that have escaped Cre-mediated excision. FACS analysis revealed that the 80% of cells lose GFP expression using 250 ng/ml of Cre recombinase non-integrating lentiviral vector. The results obtained show that Cre recombinase expression led to a significant level of transgene excision, as already reported in literature for these HLox vectors (Salmon et al., 2000, Cudrè-Mauroux et al., 2003), extending this approach to human MABs.

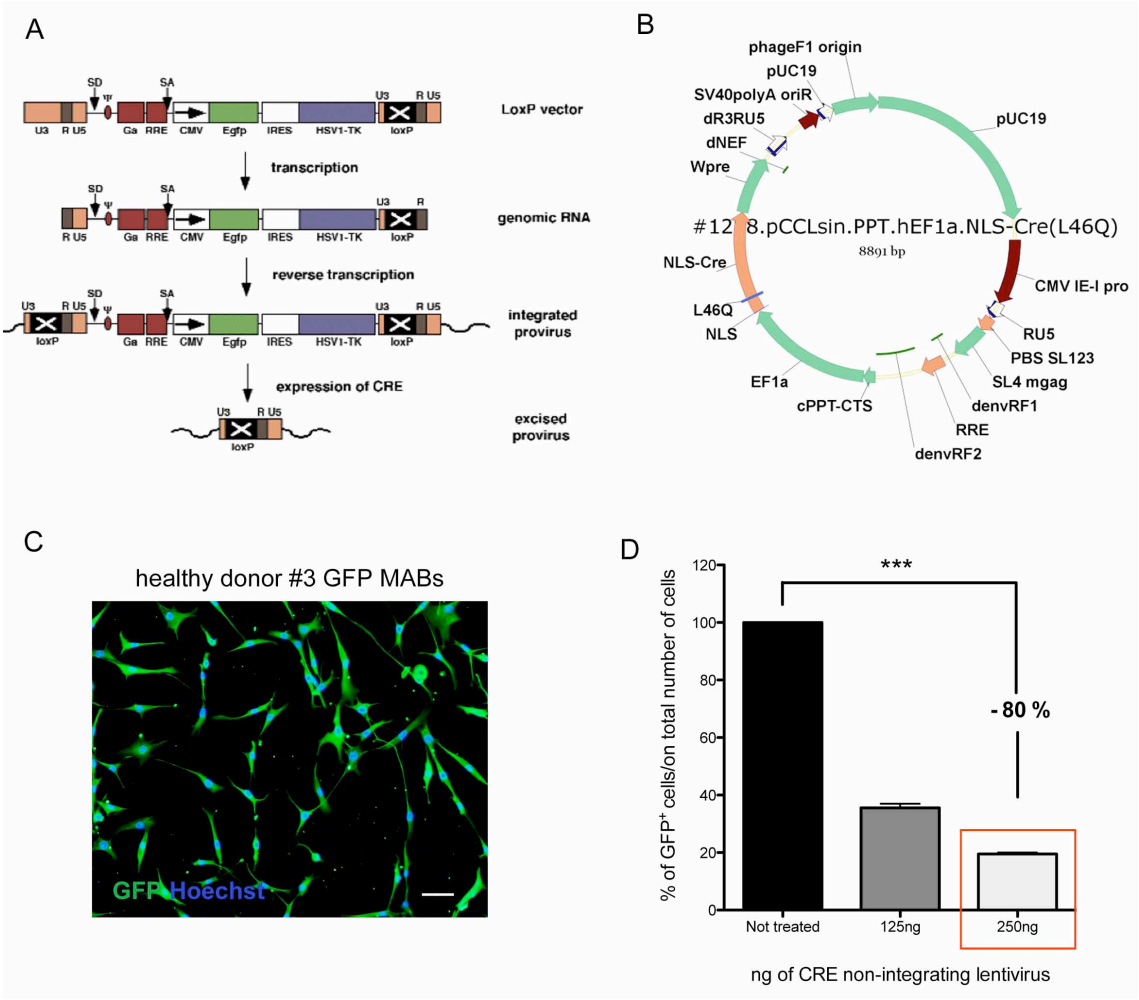


Figure 4.1.4.1 - Cre recombinase non-integrating lentiviral mediated transgene excision. A) Schematic diagram of the HLox vector life cycle. The basic elements of HIV-based vectors, i.e. LTRs, SD, SA (splice donor and acceptor, respectively), (packaging signal), Ga (fragment of gag), RRE (Rev-responsive element), have been described (Naldini et al. 1996). CMV: human cytomegalovirus immediate early promoter; EGFP: enhanced green fluorescent protein; IRES: internal ribosomal entry site of the encephalomyocarditis virus; HSV1-TK: thymidine kinase of herpes simplex virus type 1. In related constructs, Bmi-1 and hTERT cDNAs were inserted in place of EGFP. B) Map of Cre recombinase non integrating lentiviral plasmid NLS-Cre. C) Immunofluorescence showing MABs from healthy donor #3 previously infected with HLoxGFP.IRES.TK at MOI 5. EGFP is shown in green, nuclei have been stained with Hoechst. Scale bar: 50 μm D) Bar graph representation of the % of GFP positive cells remained after Cre expression using a non-integrating lentivirus as gene delivery system. ***, P< 0.0005.

4.1.5 *In vitro* myogenic potential of immortalized human MABs

Human MABs retain a variable spontaneous myogenic potential (Dellavalle et al., 2007) that physiologically decrease with *in vitro* passages (unpublished data). Despite this variability, to investigate if the immortalization process through hTERT and Bmi-1 exogenous expression could interfere with the myogenic program, an *in vitro* spontaneous skeletal muscle differentiation assay was performed culturing cells at high density in a specific differentiation medium. After ten days in differentiation medium, myotubes appeared; in particular hTERT/Bmi-1 clones differentiated with a low extent (Fig. 4.1.5.1A, top panel) similarly to their parental population (data not shown). On the other hand, hTERT/Bmi-1 healthy donor #2- and hTERT/Bmi-1 healthy donor# 3-derived MABs differentiated with a good extent (Fig. 4.1.5.1A, bottom panel), as their non immortalized counterparts did (data not shown).

To further prove their propension to myogenic conversion, hTERT/Bmi-1 healthy donor #2- and hTERT/Bmi-1 healthy donor #3-derived MABs were induced to differentiate after lentiviral transfer of MyoD-ER, a myogenesis master-gene (Choi et al., 1990) fused with estrogen receptor (ER) in order to be expressed selectively after tamoxifen administration. Five days after MyoD expression and upon serum withdrawal, multinucleated myosin heavy chain (MyHC) positive myotubes were evident, demonstrating that this two polyclonal immortalized populations were characterized by a very high myogenic conversion rate (Fig. 4.1.5.1B).

All these data demonstrate that hTERT/Bmi-1 immortalized human MABs (both clones and polyclonal populations) are able to differentiate into skeletal myotubes *in vitro,* demonstrating that the immortalization process does

not interfere *per se* with the myogenic program. Moreover, myogenic potential of hTERT and Bmi-1 expressing MABs can be also exploited via MyoD overexpression.

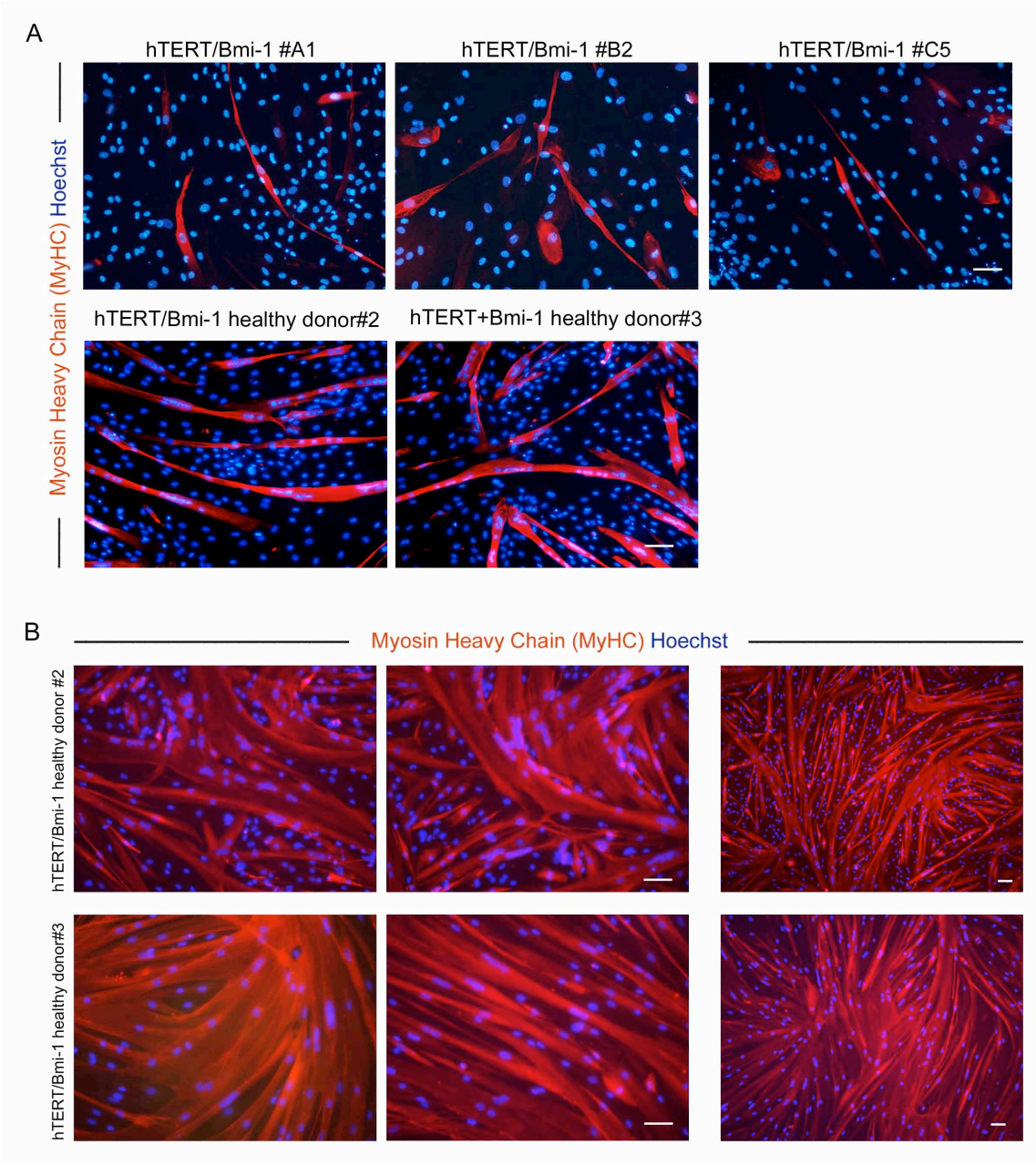


Figure 4.1.5.1 - ***In vitro*** **myogenic potential of immortalized MABs.** A) Immunofluorescence analysis of spontaneous myogenic conversion of hTERT/Bmi-1 #A1, #B2, #C5 clones and of

hTERT/Bmi-1 healthy donor#2 and #3-derived MABs. B) Immunofluorescence analysis of MyoD-ER-induced myogenic differentiation of immortalized healthy donor #2 and #3-derived MABs. MyHC in red; nuclei were stained with Hoechst. Scale bar: 50 μm.

4.1.6 *In vivo* engraftment of immortalized MABs and dystrophin expression after transplantation into *scid/mdx* mice

To investigate the *in vivo* myogenic potential of immortalized MABs, a single intramuscular (IM) injection of 5 x 10^5 of immortalized healthy donor#2-derived MABs was performed into immunodeficent dystrophic *scid/mdx* mice (choose to avoid immunorejection of human cells). Mice were killed 24 hours and three weeks after trasplantations, to evaluate respectively engraftment and their myogenic potency/dystrophin expression. 24 hours after transplantation, immunofluorescence against laminA/C (a human specific nuclear lamin) unequivocally revealed the presence of human cells (Fig.4.1.6.1A). At second instance, immunofluorescence analysis for dystrophin expression was performed three weeks after transplantation; in this case it was possible to detect dystrophin expressing fibers containing laminA/C positive nuclei, demonstrating contribution of immortalized human MABs in generating new myofibers. All these results suggest that hTERT/Bmi-1 human MABs are able to engraft the dystrophic skeletal muscle giving rise to myofibers that correctly expressed the restored dystrophin protein.

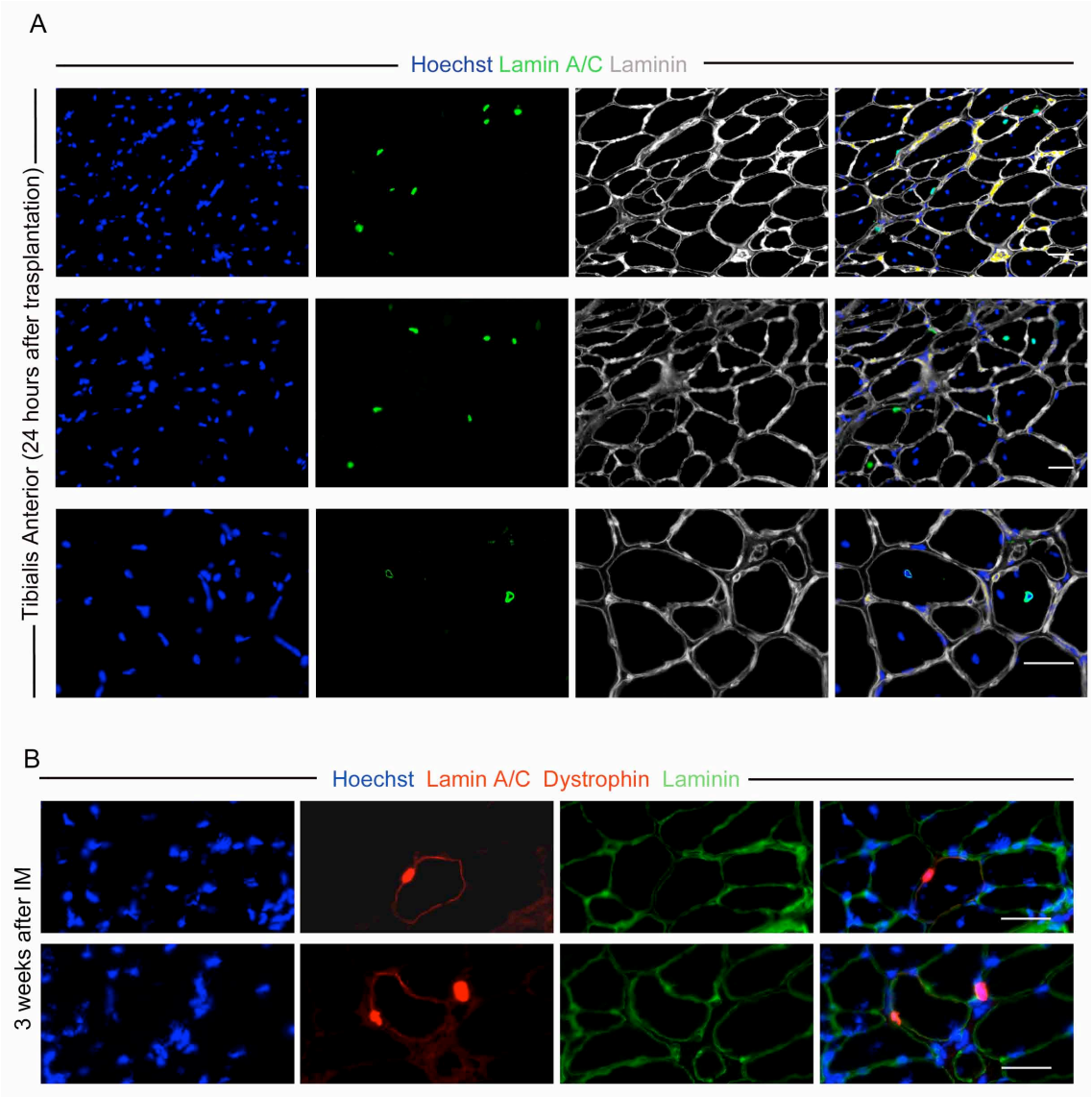


Figure. 4.1.6.1 - Intramuscular transplantation of immortalized human MABs. A) Immunofluorescence analysis for lamin A/C revealed engraftment of human cells (green) into *scid/mdx* mice skeletal muscle (Tibialis Anterior) 24 hours after a single intramuscular (IM) trasplantation. B) Immunofluorescence showing dystrophin positive fibers (red, membrane signal) generated by fusion of human laminA/C positive cells (red, nuclear signal) into dystrophic muscle (Tibialis Anterior) 3 weeks after a single IM trasplantation. Scale bar: 50 μm

4.2 IMMORTALIZATION OF DMD MABs AND GENE-CORRECTION WITH A DYS-HAC

4.2.1 Isolation and immortalization of DMD MABs

I previously demonstrated that introduction of hTERT and Bmi-1 results into an efficient immortalization of human MABs derived from healthy donors muscle biopsies. Moreover, I also showed that immortalization process does not lead to significant changes into the normal behaviour of human MABs. Even the reversibility of the system was confirmed by preliminary experiments, guaranteeing an important level of safety (see RESULTS section 4.1).

Once tested the feasibility of this experimental approach on healthy donor-derived MABs, this strategy was translated to DMD patient-derived MABs. First, as previously described, a pure AP+/CD56- MAB population from DMD patient #4 was purified through FACS-sorting (Fig. 4.2.1.1A). After initial expansion, cells were co-transduced with hTERT and Bmi-1 floxed lentiviral vectors (Fig. 4.2.1.1B). Once confirmed the presence of telomerase activity (Fig. 4.2.1.1C) and Bmi-1 protein expression (Fig. 4.2.1.1D), the myogenic potential of immortalized DMD #4 MABs was tested, demonstrating that they were still able to differentiate into skeletal muscle *in vitro* with the same extent of their parental not immortalized cells (Fig. 4.2.1.1E). All these findings confirmed what already proved for hTERT/Bmi-1 immortalized healthy donor-derived MABs, in particular that is possible to provide human MABs with an unlimited proliferative potential without causing any significant change in the normal cell behaviour.

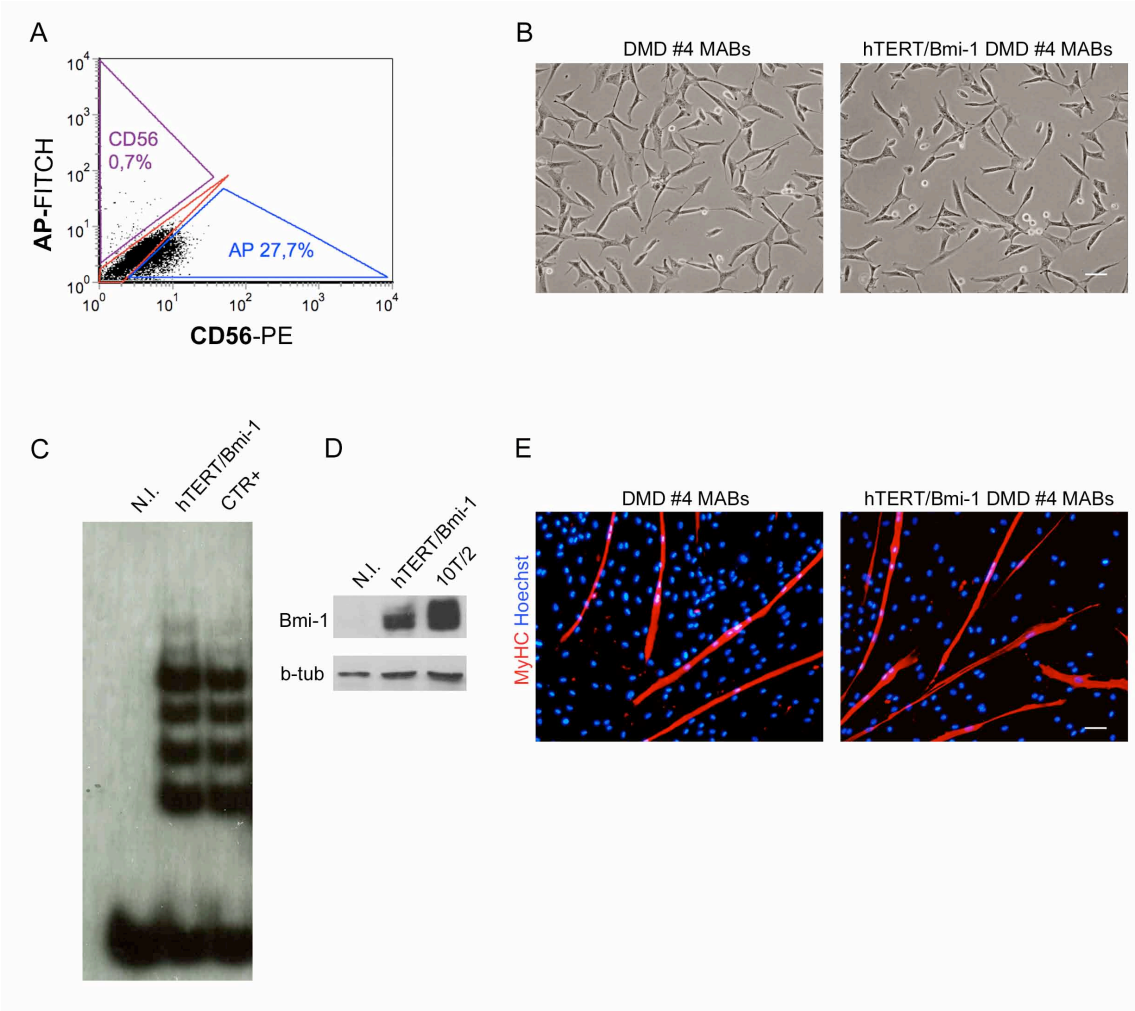


Figure 4.2.1.1 - Characterization of immortalized MABs derived from DMD patient. A) FACS-sorting analysis of a mixed population (satellite cells, fibroblasts and MABs) obtained from a muscle explant of DMD patient #4. Cells were analyzed for their positivity or negativity to AP-FITC and CD56-PE: only cells AP+/CD56- (blue selection) were sorted in order to obtain pure MAB population. B) Phase contrast morphology of DMD#4 MABs compared to hTERT/Bmi-1 DMD#4 MABs showing that introduction of hTERT and Bmi-1 did not change the typical MAB phenotype. C) TRAP assay testing presence of telomerase activity in hTERT+Bmi-1 DMD#4 MABs but not in not immortalized cells (N.I.). HeLa cells were used as positive control. D) Western Blot analysis confirming Bmi-1 expression (triplet 40-44 kDa) only in immortalized hTERT/Bmi-1 MABs. 10T/2 cells were used as a positive control and beta-tubulin (b-tub) as normalizer (50 kDa). E) *In vitro* myogenic potential of DMD#4 MABs versus

hTERT/Bmi-1 DMD#4 MABs demonstarting that immortalized cells were able to differentiate with the same extent of not immortalized cells (N.I.). Scale bar: 50 μm

4.2.2 Construction and transferring of DYS-HAC into immortalized DMD MABs

The transfer of HACs into human cells have been already demonstrated with immortalized mesenchimal stem cells (Hoshiya et al., 2009), setting the basis for doing this also in human MABs. This overall approach will provide a new autologous cell and gene therapy approach for the DMD treatment. A new DYS-HAC, called DYS-HAC2, was generated starting from DYS-HAC1 (Hoshiya et al., 2009; Tedesco et al., 2011) by homologous recombination. DYS-HAC2 presents the main outstanding features of DYS-HAC1: telomeres and centromere for autonomous replication, entire dystrophin locus for proper and functional gene-correction, selection markers and LoxP sites for cloning. On the other hand, the deletion of EGFP reporter gene lead it to be less immunogenic; moreover, the addition of neomicin resistence between FRT sites allows its removal after FLP expression. All these changes have been performed with the aim of reducing the possibility of immunoreaction against DYS-HAC itself. (Fig.4.2.2.1A).

DYS-HAC2 was then transferred into immortalized DMD #4 MABs. The tecnique used is called micro-cell mediated chromosome transfer (MMCT) which is based on the formation and fusion of small particles with one or more chromosome surrounded by nuclear and plasma membrane, which contain DYS-HAC. Chinese Hamster Ovarian (CHO) cells were used as donor cells for MMCT whereas the engineering of the HAC was performed on chicken B cells

(DT40) since they show a high frequency of homologous recombination (Fig. 4.2.2.1B).

After MMCT, immortalized DMD #4 cells have been maintained in culture for two days and then put under G418 (neomicin) selection in order to obtain DMD MAB clones containing DYS-HAC2 (DMD DYS-HAC). Three clones have been obtained from selection, # 4DYS-HAC 10, #4 DYS-HAC 11 and # 4DYS-HAC 13 (Fig.4.2.2.1C). These results demonstrate the feasibility MMCT mediated HAC transfer to immortalized DMD MABs, opening in future the possibility to translate this gene-correction stategy for the cell therapy of DMD.

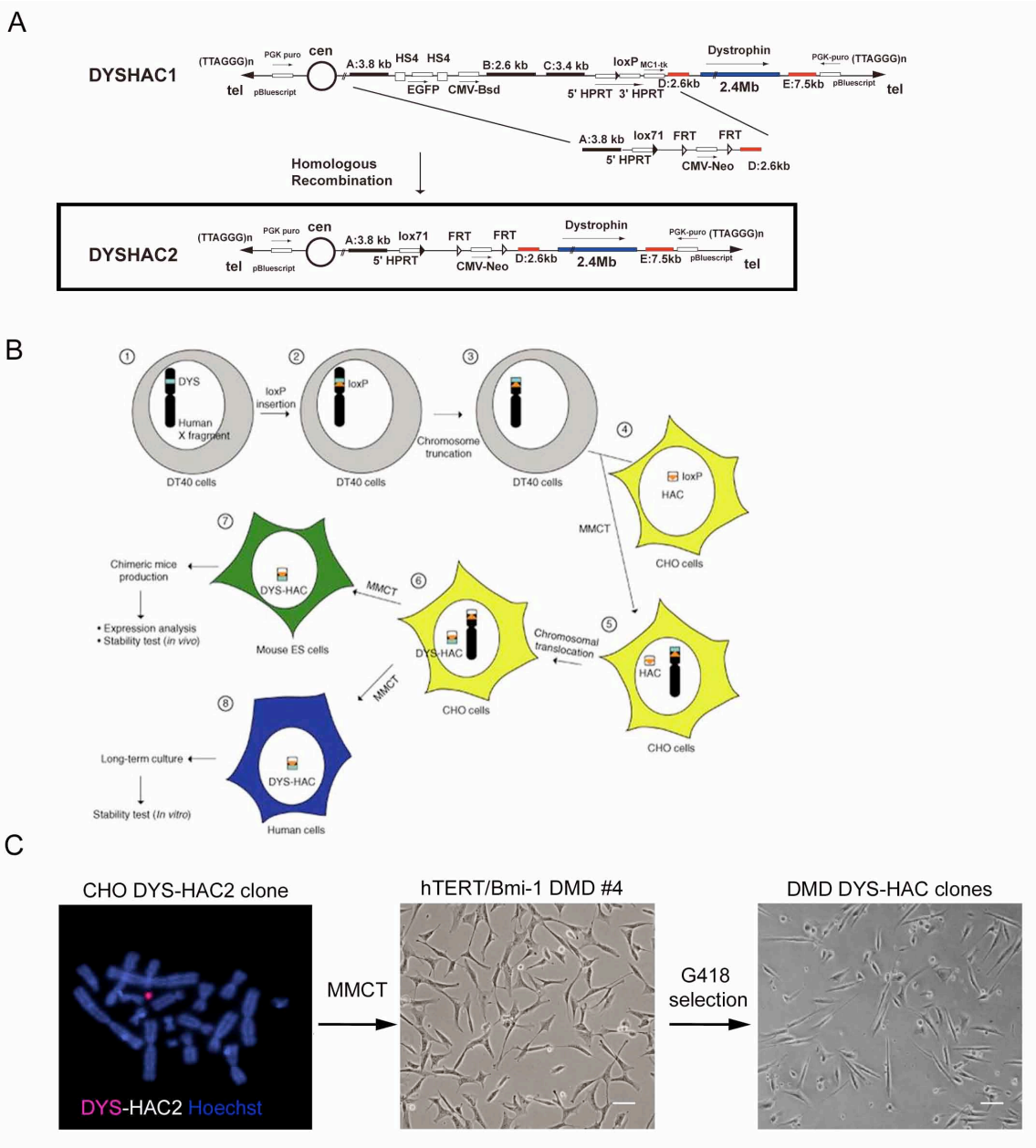


Figure 4.2.2.1 - DYS-HAC2 construction and transferring. A) Schematic map of Human Articifial Chromosome 2 containing the entire dystrophin locus (DYS-HAC2) obtained by homologous recombination from DYS-HAC1 (Hoshiya et al., 2009; Tedesco et al., 2011). The DYS-HAC1 vector contains the enhanced green fluorescent protein (EGFP) gene, the herpes simplex virus thymidine kinase (HSV-tk) gene, and several selection markers (bsd, puro- mycin, and HPRT gene). Both telomeres of the DYS-HAC are artificial. The centromere of the DYS-HAC is derived from human chromosome 21. DYS-HAC2 still has it is own centromere (cen), telomeres (tel) and loxP sites such as DYS-HAC1, but has no EGFP to reduce immunoreaction

and, moreover, neomicin (Neo) resistence is cloned between FRT sites, to allow FLP-mediated excision. B) Schematic diagram of the construction of various cells containing the DYS-HAC vector. The human dystrophin is located on the short arm of the human X chromosome. Chromosome manipulation was carried out in homologous recombination-proficient DT40 cells. To clone human dystrophin gene into the human artificial chromosome (HAC) vector using the Cre-loxP mediated chromosomal translocation, a loxP was targeted to the proximal locus of the dystrophin gene on the human X chromosome. Extra genes on the distal of the dystrophin gene were deleted by telomere-associated chromosome truncation in the DT40 cells. The modified human X chromosome fragment was transferred into Chinese hamster ovary (CHO) hybrids containing the HAC including the loxP vector by microcell-medicated chromosome transfer (MMCT). The dystrophin gene (2.4 Mb) was cloned into the HAC vector in CHO cells using Cre-loxP mediated chromosomal translocation. From the CHO hybrids, the DYS-HAC vector was further transferred to human immortalized mesenchymal stem cells (hiMSCs) and mouse embryonic stem (ES) cells. The stability of the DYS-HAC was investigated in hiMSC cells. To study the expression of the human dystrophin gene on the DYS-HAC *in vivo*, the chimeric mice were produced from the ES cells containing the DYS-HAC. C) Transferring of DYS-HAC2 into immortalized human MABs derived from DMD patient #4 through MMCT tecnique. CHO 2-12 clone was selected for MMCT after verifying the presence of DYS-HAC2 chromosome in a single and not integrated copy through fluorescent in situ hybridization (FISH) (left image); two days after transfer, cells were put under G418 (neomicin) selection (middle phase contrast image) and clones emerged three weeks after (right phase contrast image). Scale bar: 50 μm

4.2.3 Characterization of DMD DYS-HAC clones

To specifically verify the presence of the HAC and to avoid the possibility of any rearrangement, couples of primers have been designed to detect different HAC regions (1, 2, 3 and 4) and dystrophin exons (13, 14 and 18) (Fig. 4.2.3.1A) that are missing in the DMD#4 patient (which presents a deletion spanning from exon 14 to exon 20). A clone used for MMCT (CHO DYS2-12) was chosen as positive control, whereas both CHO cells (without

DYS-HAC inside) and immortalized DMD #4 polyclonal population were used as negative control. Primers to detect exon13 that is not deleted in DMD patient, and primers designed against exon 18 which is conserved between hamster and human genome has been used ss internal control. PCRs results, which are summarized below, clearly showed that all DMD #4 DYS-HAC clones were positive both for HAC regions and distrophin exons, demonstrating not only presence of DYS-HAC2 into human MABs but also a correct gene sequence (Fig. 4.2.3.1B). These results confirmed the feasibility of HACs transfer into human immortalized MABs.

Subsequently, I investigated if the HAC was present in a single and not integrated copy into the host genome. For this purpose, one of the three clones, DMD DYS-HAC #11, was selected for further analysis. As shown by FISH, the DYS-HAC2 is present in a single copy which is not integrated into human genome but remains episomal. Notably, the experiment was performed after ten passages in culture without selection, showing that the HAC is stably maintained in human dystrophic MABs. Moreover, through a metaphase spread analysis, I demonstrated that the introduction of hTERT and Bmi-1 first and of the DYS-HAC2 later, do not perturb the ploidy of human DMD DYS-HAC MABs, supporting evidences of safety of this overall approach.

To investigate the myogenic potential after DYS-HAC2 transfer, DYS-HAC#11 clone was transduced with the inducible MyoD-ER lentivirus, mainly to reintroduce myogenic potency to these cells, which might have been lost their spontaneous capacity for skeletal muscle differentiation during MMCT high-density cultures. Additionally, this step eliminated the variability in spontaneous myogenic potential observed among different mesoangioblast populations

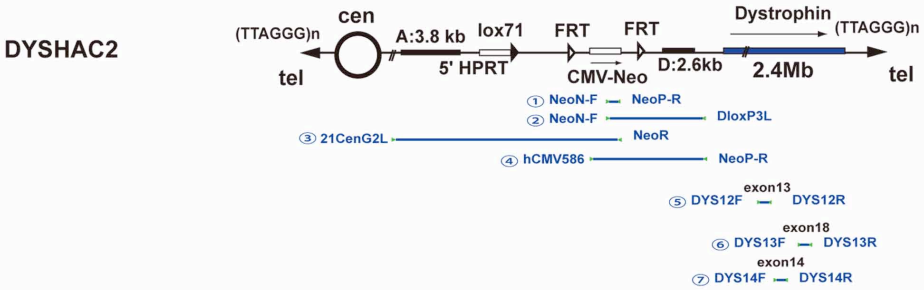
isolated from mouse (Minasi et al., 2002), human (Dellavalel et al., 2007), and canine (Sampaolesi et al., 2006) pre-and postnatal tissues.

Five days after tamoxifen induced MyoD overexpression, myotubes appeared, as demonstrated by immunofluorescence against MyHC.

In vivo tumorigenicity of this clone was tested; *scid* mice (n=4) were subcutaneously injected and, untill now (3 months) they did not develop any tumour (data not shown). It will be obviously important to follow-up these mice to confirm the absence of tumorigenic potential.

In conclusion, results demonstrate that is possible to gene-correct immortalized DMD MABs, which retain their classical behaviour.

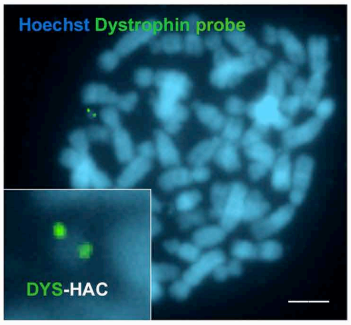
A



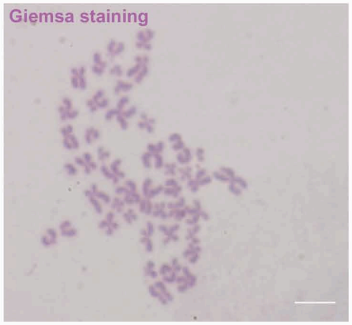
B

	Region	HAC (1)	HAC (2)	HAC (3)	HAC (4)	Exon 13	Exon 14	Exon 18
	Primer	NeoN F/NeoP R	NeoN F/DloxP3L	neor/#21cenG2L	hCMV586/DloxP3L	DYS12 F/DYS12 R	DYS14 F/DYS14 R	DYS13 F/DYS13 R
Sample	Size	0.79 Kb	4.0 Kb	7.0 Kb	4.0 Kb	152 bp	150 bp	100 bp
CHO DYS2-12		+	+	+	+	+	+	+
CHO		-	-	-	-	-	-	+
DMD#4		-	-	-	-	+	-	-
#4 DYS-HAC 11		+	+	+	+	+	+	+
#4 DYS-HAC 13		+	+	+	+	+	+	+
#4 DYS-HAC 10		+	+	+	+	+	+	+
Water		-	-	-	-	-	-	-

C



D



E

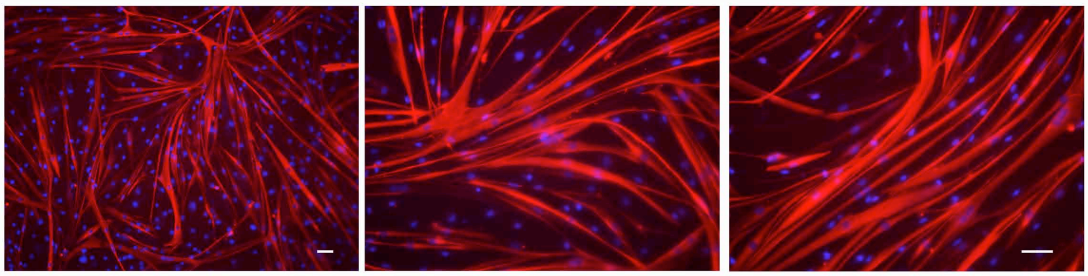


Figure 4.2.3.1 - Characterization of immortalized DMD DYS-HAC2 clones. A) Schematic map of DYS-HAC2 with couples of primers designed against different HAC (HAC1, 2, 3 and 4) and dystrophin exons (Exon 13, Exon 14, Exon 18). B) Table summarizing PCR results. PCRs were performed on DMD DYS-HAC clones obtained from MMCT on immortalized DMD #4 MABs. This patient presents a deletion from exon 14 to 20. As positive control for PCRs, CHO DYS2-12 clone used for MMCT was showed. DMD #4 immortalized MABs were used as negative control such as CHO cells. Primers used for detecting exon 18 recognized both hamster and human dystrophin exon. DMD DYS-HAC clones #10, #11 and #13 correctly presented all HACs regions. C) Example of Fluorescence In Situ Hybridization (FISH) on DMD DYS-HAC clone 11 showing the presence of DYS-HAC (green) in a single and not integrated copy. D) Giemsa staining of a representative methapase spread of the same clone showed in C). E) *In vitro* MyoD-ER induced myogenic potential of DMD DYS-HAC #11 clone both at low and high magnification. Scale bar: 50 μm

5. DISCUSSION AND FUTURE PERSPECTIVES

Several years ago, a novel population of vessel-associated progenitors, named MABs, was isolated from the dorsal aorta of mouse embryo at E9.5 (De Angelis et al., 1999; Minasi et al., 2002). These cells showed the ability to differentiate into mesodermic tissues, including skeletal muscle and, due to their origin, they managed to cross vessel walls and basal lamina of skeletal myofibers after intra-arterial delivery, representing a unique feature among myogenic cells. This allowed MABs to reach large areas of skeletal muscle tissue when injected into the arterial circulation of different DMD preclinical models, thus resulting into a functional and morphological recover of the dystrophic phenotype (Sampaolesi et al., 2003; Sampaolesi et al., 2006; Galvez et al., 2006).

In this direction, cells with a similar behaviour have been isolated from human adult muscles (Dellavalle et al., 2007) and a Phase I/II clinical trial for DMD based on allogeneic trasplantation of human MABs is currently ongoing.

On the other hand, DMD autologous cell- and gene-therapy still presents some limitations, mainly related to the large size of the dystrophin gene and to the risk of insertional mutagenesis due to random integration pattern of lentiviral vectors used for the delivery of therapeutic gene. The generation of a Human Artificial Chromosome containing the entire dystrophin locus (DYS-HAC) allowed to overcome most of these limitations (Hoshiya et al., 2009). In addition, it has been recently showed that the DYS-HAC transfer into MABs previously isolated from *mdx* mouse resulted into genetically corrected cells (mdx(DYS-HAC)MABs) able to ameliorate the dystrophic phenotype after

intra-arterial administration. This is the first evidence of a stem cell mediated gene replacement therapy with an HAC for any genetic disease (Tedesco et al., 2011).

The aim of this project was to translate this strategy into human MABs, setting the condition for a future HAC-based cell therapy of DMD.

In the case of human cells, an additional step of immortalization is necessarly required in order to extend their proliferative capability, thus preventing senescence during or after selection. Transfer of HACs in mammalian cells is inefficient (approximate frequency 10^{-5}), so selection is required to isolate clones. This is easy in mouse cells that spontaneously immortalize in culture (Katakura et al., 1998), but not in human cells, for which *in vitro* spontaneous immortalization is a rare event.

In this perspective, human MABs have been engineered to express the catalytic subunit of telomerase hTERT and the oncogene Bmi-1, as previously described for immortalization of human myoblast-derived cells (Cudrè-Mauroux et al., 2003). We first tested the immortalizing “cocktail” on human MABs derived from healthy donors. As a consequence, the first results showed that introduction of hTERT and Bmi-1 allows human MABs to bypass the early senescence which normally occurs after several passages *in vitro*, resulting in a potentially unlimited proliferative ability. This is demonstrated by the fact that all hTERT/Bmi-1 clones presented a strong proliferative rate together with a high telomerase activity, suggesting also that in human MABs the introduction of the catalytic subunit hTERT is sufficient to completely reconstitute telomerase. Moreover, BrdU incorporation assay at different passages showed that the proliferation rate of hTERT/Bmi-1 clones is maintained stable with time.

Having demonstrated the functional reconstitution of telomerase, which was stably expressed after a number of passages in culture, the next step was to verify telomerase activity on its direct biological targets, the telomeres. TRF Southern blot showed that all hTERT/Bmi-1 clones had telomeres longer compared to control clone. In particular, the ideal situation is the one of hTERT/Bmi-1 #A1 clone, which showed telomeres of a physiological and stable length, whereas hTERT/Bmi-1 #B2 and #C5 telomeres are respectively increased in length with time or slightly longer respect what described for human cells (15-25 Kilobases). By the way, this did not result into any changes of proliferation or stability of hTERT/Bmi-1 clones.

On the other hand, high telomerase activity and unlimited proliferative potential are typical features of cancer cells. To test the possibility that the immortalization process may transform MABs, *in vitro* and *in vivo* assay were performed. To assay this option *in vitro*, two classical parameters that are lost in cancerous cells, such as cell contact inhibition and growth factor dependence, have been tested. Both experiments showed that immortalized MABs are sensitive to this parameters. To enforce what emerged from *in vitro* experiments, subcutaneous injections of hTERT/Bmi-1 clones and polyclonal populations were performed into immunodeficient mice. As result, mice injected with immortalized cells did not develop any tumour.

In addition, to increase the safety of this immortalizing strategy, lentiviral vectors used for hTERT and Bmi-1 delivery have been appositely designed to present two important safety gateways. The first step consists in the Cre recombinase-mediated excision of immortalizing transgenes. The efficiency of Cre-LoxP system was tested on human MABs previously trasduced with

floxable EGFP; as delivery system a Cre non integrating lentiviral vector (NLS-Cre) was employed. As a consequence of NLS-Cre transduction, the 80% of cells did not exhibit anymore EGFP expression. Subsequently, in order to eliminate the remaining 20% of cells that have escaped Cre-mediated excision, a second safety step was introduced in lentiviral designing: TK suicide gene was cloned in IRES with the transgene between LoxP sites. This means that cells, which did not undergo transgene removal by Cre, will be killed by addition in the culture medium of ganciclovir. The assay with ganciclovir is ongoing, to set the right concentration to selectively kill target cells, avoiding the so called “by-stander” effect.

At the end, one of the most important point, is the capacity of immortalized MABs to differentiate into skeletal muscle as before, taking in account that each MAB cell line exhibited a variable myogenic extent *in vitro* and *in vivo*. Moreover, the inducible expression of MyoD into human immortalized MABs, demonstrate that is possible to strongly increase the myogenic potency of this cells. Therefore, immortalization process does not interfere *per se* with the *in vitro* intrinsic myogenic potential of human MABs whereas *in vivo* preliminary experiments to test the engraftment and the ability of human immortalized MABs together with dystrophin expression have been performed doing a single intra-muscular injection into dystrophic immunodeficient *scid/mdx* mice. In this case, it was possible to detect human cells into dystrophic skeletal muscle as well as dystrophin expressing myofibers. An extended number of *in vivo* experiments are ongoing and have been planned to better investigate not only the myogenic potential of these cells, but

also their capacity to engraft skeletal muscle upon intra-arterial injections, a MAB unique feature among myogenic cells.

Concluding this first part, it is possible to say that immortalization of human MABs has been successfully obtained through combined lentiviral-mediated expression of hTERT and Bmi-1. Moreover, hTERT expression stably reconstitutes telomerase enzymatic activity, which works properly on telomeres length, avoiding their shortening and so preventing replicative senescence. The immortalization process furthermore does not affect the typical features and behaviour of MABs, first of all their *in vitro* and *in vivo* myogenic potential, which could be also exploited via MyoD overexpression. Importantly, *in vitro* and *in vivo* assays confirm that immortalization process through hTERT and Bmi-1 expression does not lead human MABs to a tumorigenic conversion, even with the immortalizing sequences still present in the genome.

These overall results clearly suggest that introduction of hTERT and Bmi-1 is not dangerous for the genetic stability of human MABs; moreover it is possible, in case, to revert the immortalizing status of cells using a combination between Cre recombinase expression and ganciclovir administration.

The second part of this project is focused on the immortalization of DMD human MABs in order to obtain cells ready to be transferred with DYS-HAC2. For this purpose, a DMD patient was chosen and immortalized as previously described. After verifying the immortalization, DMD MABs were subjected to DYS-HAC2 transfer through MMCT. Three DMD DYS-HAC clones have been obtained upon selection; survival is a signal of the DYS-HAC2

presence, since selection resistance was conferred by HAC itself. Moreover, PCRs performed with different couples of primers designed to detect both HAC regions and dystrophin exons, confirmed DYS-HAC2 presence and avoid the possibility of rearrangements. These results show that is possible to successfully transfer HACs into DMD MABs, obtaining genetically corrected cells and that, after immortalization, they are able to survive during and after selection. In addition, a FISH assay, using a probe which recognize a portion of dystrophin that is deleted into DMD patient used for this study, showed that DYS-HAC2 is present in a single and not integrated copy into the host genome, whereas metaphase chromosome spread analysis, demonstrated that introduction of hTERT and Bmi-1 and then, of DYS-HAC2 did not lead to chromosomal instability.

After MMCT high density cell cultures, the myogenic potential of DMD DYS-HAC clones was restored after transduction with a MYOD-ER lentiviral vector. As a matter of fact, after MyoD induction with tamoxifen, cells were able to differentiate *in vitro* with a high extent, showing so they retained an intrinsic myogenic potential. Future experiments will be planned to verify the *in vivo* myogenic potential of these cells in ameliorating the dystrophic phenotype together with deeper investigations on safety.

All these results taken together strongly support the feasibility of a new autologous cell therapy approach to treat DMD, based on trasplantation of DYS-HAC genetically corrected DMD MABs. The use of HACs as tool for the gene delivery could change the field of autologous cell theraphy, in particular for this pathology. If successful, this strategy would present many advantages over

conventional gene-correction systems, among these the avoiding of risk of insertional mutagenesis, an unlimited space for genes and cDNA that could be engineered in the HAC, no patient selection for mutation type and no immune-suppression needing.

Moreover, HACs can be further engineered to carry on additional genes with an improving effect on dystrophic phenotype such as MyoD, IGF-1 (Musaro, 2005), PlGF (Gargioli et al., 2008), HSV-TK, MyoD-ER. In this direction, in our laboratory we are developing new-generation DYS-HAC vectors able to carry also floxed immortalizing sequences, thus completely avoiding the use of integrating lentiviral vectors.

Currently, different experimental therapies are entering clinical trials but so far only exon-skipping promises a solution to treat DMD. On the other hand, also if successful exon-skipping is a mutation-specific gene-correction strategy, so a consistent number of mutations remained impossible to be corrected. In this case, transplantation of DYS-HAC genetically corrected human cells, may represents the best solution.

6. MATERIALS & METHODS

6.1 ISOLATION FROM MUSCLE EXPLANTS AND CULTURE OF HUMAN MABs

Cells were prepared from healthy donors and dystrophic patient (DMD) undergoing diagnostic biopsy (and later classified as non affected by secondary myopathies) and maintained in culture as previously described (Tonlorenzi et al., 2007). Briefly, each muscle sample was stored in DMEM w/o FCS, with antibiotics and kept at 4 °C for a maximum of 24 hours before dissection. It was then rinsed in PBS without Ca_2-$Mg_2$and sharply dissected into 1–2 mm diameter pieces with a scalpel. Fragments were transferred to a Petri dish coated with type I collagen (1 mg ml–1 in 0.1 M acetic acid). The medium consisted of MegaCell DMEM (Sigma, St Louis, MO) supplemented with 5% FBS, 5ng/ml basic fibroblast growth factor (bFGF), 2 mM glutamine, 0.1 mM β - mercaptoethanol, 1% non essential amino acids, 100 IU/ ml penicillin and 100 mg/ml streptomycin. The tissue fragments were cultured for 7–8 days. After the initial outgrowth of fibroblast-like cells, small round and refractile cells were observed. Because of their poor adhesion (many of these cells were floating), cells were collected by gently pipetting and plated on plastic with the MegaCell supplemented medium in low oxygen (5% O_2 , 5% CO_2) incubators to avoid oxidative stress.

6.2 CELL SORTING OF MABs

The cells obtained from the muscle explants were a mixed population of MABs, and fibroblasts. To select MABs, a Cell sorter (DIVA Vantage, BD) was used to purify them (AP+/CD56-) from myoblasts (AP-/CD56+) and fibroblasts (AP-/CD56-). In order to do this, cells were harvested and resuspended in a solution containing 1% FBS and 2mM EDTA in PBS and incubated with AP-FITC (Santa Cruz) and CD56-PE (Milteny Biotec) antibody for 30 minutes at 4°C and then analyzed. A primary gate based on physical parameters (forward and side light scatter, FSC and SSC, respectively) was set to exclude dead cells or small debris. Then cells were analyzed for the expression of AP versus CD56. Only AP+/CD56- cells were collected to have a pure MABs population.

6.3 LENTIVIRAL PRODUCTION, TITRATION AND TRANSDUCTION

Lentiviral production was performed as previously described (Messina et al., 2010). Briefly, lentiviral particles were produced by transient transfection of the vector of interest in association with the packaging vectors (pREV, pD8.74 and pVSV-G) in HEK293T. After 30 hours, culture medium from transfected cells was filtered with a 0.45 mm filter and 100-times concentrated after centrifugation at 20,000 rpm for 2hrs (at 20°C). Working concentrations were determined by titration on HeLa cells and processed through quantitative real time analysis of viral sequences.

For integrating lentiviral transductions, human MABs were plated at a

density of 10^5 cells. When cells reached 70-80% of confluence they were transduced. In detail, cells were incubated overnight (O/N) with the viral dilution in a final volume of 1 ml with polybrene, to increase infection efficiency. The day after, cells were rinsed in PBS and cultured with normal growing medium.

In the case of transduction with non-integrating lentiviral vectors, human MABs were plated at density of 10^5 cells and incubated with 125 ng or 250 ng of virus in 1 ml suspension for 24 hours in presence of polybrene. The day after cells were rinsed in PBS and cultured with normal growing medium.

The lentiviral vectors used for this study are the following: HLox.CMV.hTERT.IRES.TK, Hlox.CMV.Bmi-1 and Hlox.CMV.EGFP.IRES.TK (Salmon et al., 2000); MyoD-ER construct was kindly provided by Dr. Jeffrey S. Chamberlain (University of Washington School of Medicine, Seattle, USA) and used as previously described (Kimura et al., 2008); NLS-Cre non integrating lentiviral vector was designed, produced and titered by Angelo Lombardo (unpublished).

6.4 CELL CLONING

Cells were counted in a haemocytometer. The cell suspension was cloned by limiting dilution or by cell sorting facility to have single cell per dish.

6.5 TELOMERASE ACTIVITY ASSAY (TRAP)

To evaluate the telomerase activity, TRAPeze® Telomerase Detection Kit (Chemicon) was used. Cells were collected and telomerase was extracted with CHAPS lysis buffer. The TRAPeze kit is a one buffer, two-enzyme system utilizing the polymerase chain reaction (PCR). In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto 3' end of a substrate oligonucleotide (TS) at 30°C for 30 minutes. In the second step, the extended products are amplified by PCR (33 cycles) using the TS and reverse (RP) primers generating a ladder of products with 6 base increments starting at 50 nucleotides, each one corresponding to a telomeric repeat added by telomerase (i.e., the number of bands revealed on gel will then correlate with the telomerase activity).

The PCR products were run on 10% non-denaturing polyacrylamide gel at 400V for 3 hours; the gel was then directly exposed with X-ray film. For additional details see TRAPeze Telomerase Detection Kit datasheet.

6.6 WESTERN BLOTTING

Proteins for western blot were extracted from cells using LEMLI buffer with 2% sodium dodecyl sulphate (SDS), 50mM Tris-HCl ph6.8, and 10% glycerol. Protein concentrations were determined by BCA protein assay (Pierce) using bovine serum albumin as standard and after determination DTT (dithiothreitol) was added. 50µg of proteins were loaded and separated by 8%

SDS polyacrylamide gel electrophoresis (SDS-PAGE) to detect Bmi-1 (triplet 40-44 kDa). Proteins were then transferred to Amersham membranes (1hr at 150mA), saturated with 5% milk in 0.1% Tween-20 (Sigma)-PBS and hybridized overnight at 4°C with Bmi-1 antibody (Millipore). The filters were then washed four times (10 minutes each at RT) with 0.1% Tween-20 (Sigma)-PBS and reacted with the proper HRP-conjugated IgGs (Amersham; 1:10000 dilution) for 45 hour at RT, washed four times and finally visualized with the ECL immunoblotting detection system (Amersham). Mouse anti-beta-tubulin (50 kDa, Covance) and anti-GAPDH (27 kDa, Sigma) were used to normalize.

6.7 POPULATION DOUBLING CURVE

The proliferation ability of clones was evaluated plating 10^5 cells per clone. Every time cells reached 70-80% of confluence they were collected, counted with haemocytometer and expanded. Growth curves were obtained calculating Population Doubling (PD) as proliferation index. PD = logN/log2; N= cells collected/cells plated.

6.8 BrdU INCORPORATION AND DETECTION

To test BrdU (5-bromo-2-deoxiuridine) incorporation, 8 x 10^4 cells were plated. 24 hours after, cells were incubated 1 hour with 50μM BrdU diluted in standard growing medium. After incubation cells were fixed with Ethanol 95%,

Acetic acid 5% and then incubated 20 minutes with 1.5M HCl. Immunofluorescence staining was performed with Anti-BrdU detection kit (Amersham) according manufacturer's instruction and nuclei were stained with Hoechst. BrdU+ and Hoechst+ cells were counted at fluorescence microscopy. The proliferation rate was calculated as percentage of BrdU+ cells on total number of nuclei (Hoechst+ cells).

6.9 TELOMERIC RESTRICTION FRAGMENTS ASSAY (TRF)

DNA was extracted from cells with Blood & Cell Culture DNA Mini kit (Qiagen) and TRF assay was performed with TeloTAGGG Telomere Length Assay Kit (Roche) according manufacturer's instructions. Briefly, purified genomic DNA was digested by an optimized mixture of frequently cutting restriction enzymes (Hinf1 and Rsa1). The sequence specificity of these enzymes ensures that telomeric DNA and sub-telomeric DNA are not cut, while non-telomeric-DNA is digested to low molecular weight fragments. Following DNA digestion, the genomic fragments were separated by 0.8% gel electrophoresis at 5V/cm for 2-4 hours and transferred by capillarity to a nylon membrane. The blotted DNA fragments are hybridized to a digoxigenin (DIG)-labeled probe specific for telomeric repeats and incubated with a DIG-specific antibody covalently coupled to alkaline phosphate. Finally, the alkaline phosphatase on the antibody metabolizes CDP-Star, a highly sensitive chemiluminescent substrate; this produces a visible signal that indicates the location of the immobilized telomere probe (and, hence, the TRF) on the blot. The average TRF length can be

determined by comparing the location of the TRF on the blot relative to a molecular weight and to a standard control. The signal responses were analyzed by a computer-assisted system derived from NIH Image 1. For detailed information see datasheet.

6.10 CELL CONTACT INHIBITION ASSAY

8×10^4 cells were plated in quadruplicate. BrdU incorporation was first assayed when cells were subconfluent (60-70%; baseline). Then BrdU incorporation rate was tested 4 days, 8 days and 12 days after the beginning of the experiment, when cells were left to go to confluence. HeLa cells were used as control.

6.11 GROWTH-FACTOR DEPENDENCE ASSAY

8×10^4 cells were plated in quadruplicate and BrdU incorporation was assayed to verify the proliferation rate in standard culture conditions (baseline). The day after cells were shifted in a growth factor and serum-free medium and BrdU incorporation was performed at different time point, 4 days, 8 days and 12 days in growth factor and serum-free medium. HeLa cells were used as control.

6.12 *IN VIVO* TUMORIGENIC ASSAY

Four *scid* immunodeficient mice/clone or /polyclonal population (n=24) were injected subcutaneously in the dorsal flank with 2 x 10^6 cells/200 µl of PBS (without calcium and magnesium) containing 0.2 international units of sodium heparin (Mayne Pharma, Australia). The mice are followed-up to investigate the capability of hTERT/Bmi-1 clones to give rise tumour formation. As technical positive controls, *scid* injected with HeLa cervical carcinoma cells were used (*n* = 3/3 mice).

6.13 FLOW CYTOMETRY

Cells were harvested and resuspended in 1 ml of a solution containing 1% FBS and 2mM EDTA in PBS. After PBS washing, cells were fixed in 2% PFA before FACS analysis. Analysis was performed on at least 10.000 events for each sample and determined using a FACScalibur flow cytometer (Becton Dickinson: BD). The acquisition was performed using CELLQUEST software (BD) and analyzed using FCS-express software. A primary gate based on physical parameters (forward and side light scatter, FSC and SSC, respectively) was set to exclude dead cells or small debris.

6.14 *IN VITRO* MYOGENIC DIFFERENTIATION ASSAY

Skeletal myogenic differentiation of human MABs was induced by plating cells at confluence onto matrigel coated dishes and exposed to DMEM supplemented with 2% horse serum (EuroClone) from a minimum of 7 to a maximum of 10 days. When MyoD-ER was used, cells were exposed to 1μM of 4-hydroxy-tamoxifen (4OHT; Sigma) for 24 hours and then switched to differentiation medium containing DMEM plus 2% horse serum and 4OHT.

6.15 IMMUNOFLUORESCENCE

Cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma) at room temperature (RT) for 10 minutes, permeabilized with 0.2% Triton X-100 (Sigma) and 1% BSA (Sigma) in PBS for 30 minutes at RT and 10% donkey and/or goat serum (Sigma) was used as blocking solution to reduce secondary antibody background signal. Muscle samples were frozen in liquid nitrogen cooled isopentane and serial 7 μm sections were cut with a cryostat (Leica). Cells and tissue sections were incubated overnight at 4°C with the following primary antibodies: mouse anti-dystrophin Dys1, Dys2 (Novocastra); rabbit anti-laminin (Sigma); mouse anti-myosin heavy chain (MyHC; MF20, Hybridoma Bank rabbit anti-GFP (Chemicon); chicken anti-GFP (Millipore); mouse anti-laminA/C (Novocastra).

After incubation, samples were washed with 0.2% Triton X100, 1% BSA in PBS and then incubated with the appropriate 488, 546, 594 or 647-

fluorochrome conjugated IgGs (Molecular Probes) together with Hoechst dye for 1 hour at RT in 0.2% Triton X100-PBS. After three final washes, dishes or slides were mounted using mounting medium (Dako) and watched under fluorescent microscopes (Nikon and Leica). Images were analyzed using PhotoshopCS (Adobe) software.

6.16 INTRA-MUSCULAR TRASPLANTATION

3 weeks old *scid/mdx* mice were used for *in vivo* experiment. Mice were anesthetized with intra-peritoneal avertin (Sigma) in 0,9% saline. Intra-muscular delivery was done by injecting 10^5 cells diluted in 50 µl of PBS without calcium and magnesium into Tibialis Anterior muscles using a 30G syringe (BD).

6.17 CONSTRUCTION AND GENERATION OF DYS-HAC2

The DYS-HAC vectors were constructed from a HAC backbone named 21HAC2 and details about the procedure are available in reference (Kazuki et al., 2011). The targeting vector including two 3.8 kb and 2.6 kb fragments for homologous arms corresponding to human chromosome 21 and X locus in AL050305 and AP001657 (Hoshiya et al 2009, Kazuki et al 2010), pN for introducing 5′ HPRT-lox71/FRT-Neo-FRT and deleting extra genes on DYSHAC1, was constructed in the pBSII backbone vector (Stratagene) using standard ligation technique (Hoshiya et al, unpublished).

6.18 METAPHASE CHROMOSOME SPREAD AND FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

To block cell into metaphase, human MABs were incubated overnight at 37°C with 0.2 µg/ml Demecolcine (Sigma), trypsinized and incubated with 0.075M KCl. After three washes with Carnoy's solution (three parts of methanol, one part of acetic acid), one drop of cell suspension was spreaded on each microscope slide (pre-incubated in 50% ethanol). Cells were fixed on the slides with a Bunsen's burner and kept for one week at -80°C. Chromosomal DNA was counterstained with Hoechst dye (Sigma) or with Giemsa staining and images were captured using a Leica (Germany) DMI6000B microscope equipped with a AF6000 system and LAS AF 2.3.5 software. A minimum of 20 metaphase per experiment was counted. FISH analyses were performed onto fixed metaphase spreads of each CHO or DMD hTERT/Bmi-1 clone using the biotin-labelled bacterial artificial chromosome (BAC) containing a human dystrophin region (cat. number RP11-954B16, from Chori, Children's Hospital Oakland Research Institute).

6.19 MICROCELL MEDIATED CHROMOSOME TRANSFER (MMCT)

MAB hybrids containing the DYS-HAC were produced by MMCT technology from a donor Chinese hamster ovary (CHO) cells containing the DYS-HAC2 and maintained with 0.8 mg/ml G418 (Sigma). The DYS-HAC CHO hybrids were obtained from DT40 cells hybrids previously described (Hoshiya et

al., 2009). Briefly, the DT40 hybrids containing a single copy of a human X chromosome fragment (long arm deletion of X chromosome) were generated by MMCT from mouse A9 cells containing this fragment (Hoshiya et al., 2009) and maintained in culture in Roswell Park Memorial Institute (RPMI) medium 1640 (Invitrogen) containing 10% FBS, 1% chicken serum (Invitrogen), 50μmol/l 2-mercaptoethanol (Sigma), 100 IU ml^{-1} penicillin and 100 mg ml^{-1} streptomycin and G418 (Invitrogen) selection (1.5mg/ml). The modified X fragment containing the human dystrophin gene was transferred from the DT40 hybrids into the CHO hybrids containing the HAC vector by MMCT. Microcells were prepared by centrifuging 1×10^9 DT40 cells attached on flasks (Nunc) coated with poly-L-lysine (Sigma) and were then fused with 1×10^6 CHO cells using 47% polyethylene glycol (PEG) 1000 (WAKO). CHO hybrids were selected in 0.8 mg/ml G418 and picked for expansion. After construction of the HAC vector containing the entire human dystrophin gene (DYS-HAC) by site-specific translocation in CHO hybrids, transfer of the DYS-HAC2 vector from CHO cells to immortalized DMD MABs was performed using standard procedures (Hoshiya et al., 2009): target cells (hTERT/Bmi-1 DMD MABs) were PEG-fused with microcells prepared from CHO DYS-HAC 2-12 clone, screened by FISH. Finally, DMD DYS-HAC clones obtained were characterized by PCR (see **MATERIALS & METHODS** section **6.20**) and FISH (see **MATERIALS & METHODS** section **6.18**)

6.20 PCRs

HACs regions of DYS-HAC2 vector were detected using the following primers and PCR conditions:

- NeoN-F 5'- aaggaaaagctagcgccaccatgattgaacaagatggattgcac -3'
- NeoP-R 5'-aaggaaaaaagtttaaactcagaagaactcgtcaagaag-3'
- DloxP3L 5'-gcatgggggaggagagaagagagatgta-3'
- NeoR 5'-tgatcgacaagaccggcttcca-3'
- #21CenG2L 5'-ctctaccattagaatggaaacgtcatc-3'
- hCMV586 5'-cgtaacaactccgccccatt-3'

Primers conditions:

98°C, 1 minute

98°C, 15 seconds

68°C, depends on product size* 35 cycles

72°C, 10 minutes

- NeoN-F/NeoP-R size 0.79 Kb, * 1 min
- NeoN-F/DloxP3L size 4 Kb, * 4,5 min
- 21CenG2L/NeoR size 7 Kb, * 7,5 min
- hCMV586/NeoP-R size 4 Kb, * 4, 5 min

Human Dystrophin of DYS-HAC2 has been detected using following primers and PCR conditions:

- DYS13F 5'-caatccatgggcaaactgta-3',
- DYS13R 5'-ctgtgctgtactcttttcaagttttt-3'
- DYS14F 5'-ctgaaagagttgaatgactggcta-3',

- DYS14R 5'-cttggcgttttaggtcttcaa-3'
- DYS12F 5’-ggccgggttggtaatattct-3’
- DYS12R 5’-ttgctttgtttttccatgct-3’

Primers conditions:

94°C, 5 minutes

94°C, 1 minute

56°C, 1 minute

72°C, 1 minute 35 cycles

72°C, 5 minutes

- DYS13F/13R size 150 bp (exon 18)
- DYS14F/14R size 100 bp (exon 14)
- DYS12F/12R size 152 bp (exon 13)

6.21 MICE

Scid/mdx (C57BL/6 background) mice were housed both in Charles River Laboratories, Calco Italy and in San Raffaele Scientific Institute animal house. All mice were kept in specific pathogen free (SPF) conditions and all procedures involving living animals conformed to Italian law (D.L.vo 116/92 and subsequent additions) and were approved by the San Raffaele Animal Care and Use Committee (IACUC number 355).

6.22 STATISTICAL ANALYSIS

Values were expressed as means ± s.e.m. Significance of the differences between means was evaluated by two-tailed Student's t-test. $P < 0.05$ was considered to be statistically significant. Data were analyzed using GraphPad Prism 5.

- HAC: human artificial chromosome
- H&E: hematoxylin and eosin
- HGF: hepatocyte growth factor
- hMADS: human multipotent adipose-derived stem cells
- HSC: hematopoietic stem cell
- HSV-TK: herpes simplex virus thymidine kinase
- hTERT: human telomerase reverse transcriptase
- IF: immunofluorecence
- IGF1: insulin-like growth factor 1
- MAB: mesoangioblast
- MAPC: multipotent adult progenitor cell
- MD: muscular dystrophy
- MDSC: muscle-derived stem cell
- Mdx: X chromosome-linked MD (DMD mouse model)
- mdx(DYS-HAC)MAB: mdx-derived MAB containing the DYS-HAC
- MMCT: microsome-mediated chromosome transfer
- MSC: mesenchymal stem cell
- Myf5: myogenic factor 5
- MyHC: myosin heavy chain
- MyoD: myogenic differentiation 1
- MyoD-ER: MyoD and ER fusion protein
- NK: natural killer
- NO: nitric oxide
- Pax3: paired-box 3
- Pax7: paired-box 7

- PCR: polymerase chain reaction
- PDGFR: platelet-derived growth factor receptor
- PSC: pluripotent stem cells
- rAAV: recombinant AAV
- RNA: ribonucleic acid
- RT-PCR: reverse transcription PCR
- SC: satellite cell
- Scid: severe combined immune-deficiency
- SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
- Sgca: alpha-sarcoglycan
- SP: side population
- TA: tibialis anterior
- TGFβ: transforming growth factor beta
- X-Gal: also abbreviated BCIG (bromo-chloro-indolyl-galactopyranoside)
- WB: Western blot

7. LIST OF ABBREVIATIONS

- 4OHT: 4-hydroxy-tamoxifen
- AAV: adeno-associated vector
- ANOVA: analysis of variance
- AON: antisense oligonucleotide
- AP: alkaline phosphatase
- aSMA: alpha-smooth muscle actin
- bFGF: basic fibroblast growth factor
- BMD: Becker muscular dystrophy
- BMT: bone marrow transplantation
- CHO: Chinese hamster ovary
- DMD: Duchenne muscular dystrophy
- DMEM: Dulbecco's modified Eagle's medium
- DNA: deoxyribonucleic acid
- DYS-HAC: dystrophin-HAC
- EBD: Evans blue dye
- EPC: endothelial progenitor cell
- ER: estrogen receptor
- ESC: embryonic stem cell
- FACS: fluorescence-activated cell sorting
- FBS: fetal bovine serum
- FISH: fluorescence in situ hybridization
- GAPDH: glyceraldehyde 3-phosphate dehydrogenase
- GFP: green fluorescent protein

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